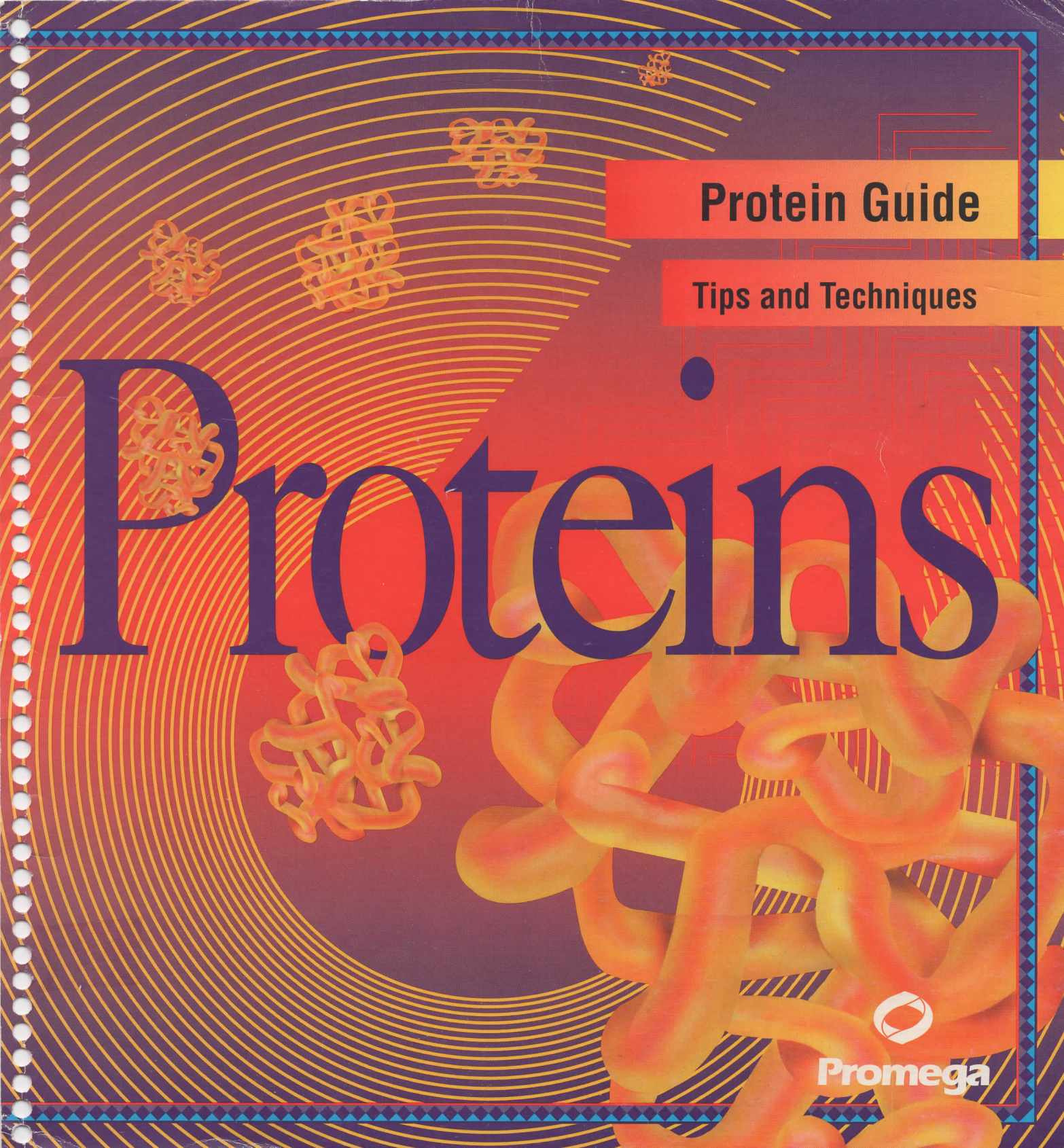
Protein Guide

Tips and Techniques

Proteins



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Promega has established itself as a provider of superior technical support for its products through its Technical Services Department and literature such as the *Protocols and Applications Guide*. With the *Protein Guide: Tips and Techniques*, we hope to continue our tradition of quality technical support by describing protein techniques useful in solving molecular biology questions.

The *Protein Guide* takes a different approach to providing technical information than literature either previously developed by Promega and others. Instead of providing detailed protocols like those supplied with our products, the *Protein Guide* presents the rationale for the selection of a particular technique, the general steps involved in the technique, sources of problems with the technique and possible conditions to optimize the technique. Much of this information is presented in tabular or bulleted form so it can be easily accessed.

The information presented in the *Protein Guide* may be considered analogous to the technical advice received from a colleague familiar with a technique. Most importantly, they would give you their evaluation of the strengths and weaknesses of the technique based on their experience, along with references to protocols. These types of discussions assist in the decision of whether the technique is likely to answer a particular research question and often provides "tips for success" that are not easily obtained from a review of the publications. These talks also assist in the development of strategies to adapt the technique to a particular experiment.

The topics chosen are ones which we find most helpful in our work developing and manufacturing products. They include:

- Electrophoresis and staining of proteins
- Immunoblotting techniques
- Strategies for structural analysis
- *In vitro* translation systems
- General strategies for protein purification and protein refolding

In addition to the background information on these techniques, we provide some common applications of the technique at the beginning of each chapter. The chapters also include numerous scientific references for those in search of more information, along with other Promega technical literature on the topic.

We hope the *Protein Guide* provides answers to the many questions related to setting up a new technique. In addition, we hope the *Protein Guide* becomes a valuable reference tool for a variety of options available to solve numerous research questions. We welcome your input on how we can expand and improve this information in the future.

Promega Protein Guide: Tips and Techniques

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Part Number AP003

The Promega *Protein Guide: Tips and Techniques* is printed on recycled paper.

800-356-9526

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399 USA
Telephone 608-274-4330
FAX 608-277-2516
Telex 62057092

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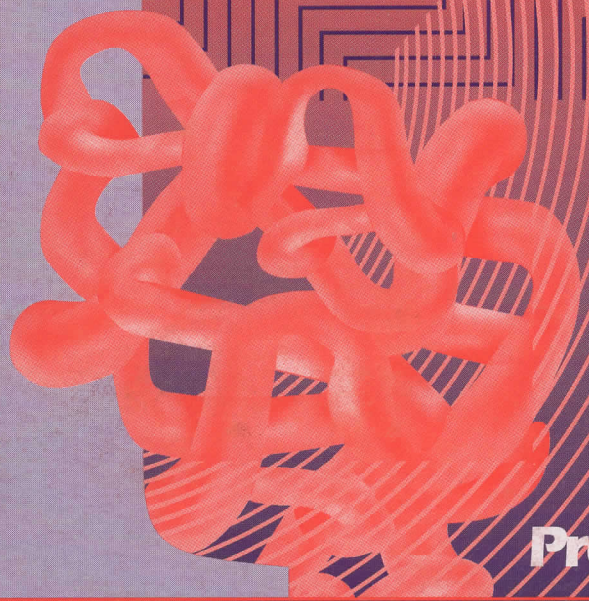
Introduction

Protein Guide



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Introduction

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Introduction

I. Suggested Protein Techniques

While this guide may be useful to anyone studying proteins, we have attempted to focus on protein techniques that can help solve some problems that many molecular biologists face. A summary of some of these problems is presented below with suggested protein techniques which may resolve the problem.

Molecular Technique	Problem/Question	Protein Technique	Chapter
Cloning			
Producing cDNA libraries	Purity of mRNA.	*In vitro* translation.	5
Probes for screening libraries	No antibody available for screening.	Proteolytic or chemical digest of small amount of protein followed by protein sequencing to generate DNA probe.	4
Verifying clones	Does the clone contain a full-length gene?	*In vitro* translation.	5
		Immunoblotting	3
		Proteolytic digestion and comparison to native protein.	4
	Is it expressed but toxic to cells?	*In vitro* translation in wheat germ, reticulocyte lysates, or *E. coli* extracts.	5
	Is the cloned protein similar to proteins from other species?	Proteolytic digests.	4
	Will the expressed protein be active?	*In vitro* translation.	5
Mutagenesis	Rapid screening for mutant activity.	*In vitro* translation.	5
	Activity/domain relationships.	Proteolytic digests.	4
Epitope Mapping	Identification of region of antibody recognition.	Immunoblotting.	3
	Production of truncated gene products.	*In vitro* translation in Linear S30 Extract or TnT Lysates.	5
Post-Translational Modifications	Where are the potential glycosylation/signal sequences?	*In vitro* translation in rabbit reticulocyte lysate with microsomal membranes.	5
	What is the location of a modification on a protein?	Proteolytic digest.	4

I. Suggested Protein Techniques

(continued)

Molecular Technique	Problem/Question	Protein Technique	Chapter
Protein Expression			
	Expression in inclusion bodies.	Protein purification and refolding techniques.	7
	Should the protein be expressed in another expression system?	Purification of soluble proteins from *E. coli.*	6
	Expressed protein sensitive to proteases.	*In vitro* translation.	5
	Expression of PCR* products.	*In vitro* translation in S30 Linear Extract or TNT Lysate.	5
	Developmental or tissue specific expression.	Immunoblotting.	3
DNA/RNA/Protein Interactions			
	Quick screen for binding activity.	*In vitro* translation followed by gel shift assay.	5
Protein/Protein Interactions			
	Do proteins interact?	Co-expression of proteins using *in vitro* translation.	5

*The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202, assigned to Hoffman-La Roche. Patents pending in other countries.

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Electrophoresis

Basic Techniques

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Electrophoresis/Staining of Proteins

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Electrophoresis/Staining of Proteins

I. Gel Preparation

Fractionation of proteins in polyacrylamide gels is one of the primary means of their characterization because of its speed and ease of use. Many methods to separate both native and denatured proteins exist in the scientific literature (for review, see ref. 1). The most widely used technique, however, is SDS polyacrylamide denaturing gel electrophoresis (SDS-PAGE) (2), which will be discussed here.

The goal of SDS-PAGE is to separate proteins in a complex mixture solely on the basis of molecular weight. First, the protein sample is denatured with heat in the presence of SDS and a reducing agent, usually β-mercaptoethanol. The SDS coats the proteins, providing them with a net negative charge proportional to their length. When the coated sample is run on an SDS polyacrylamide gel, the proteins separate by charge and by the sieving effect of the gel matrix. Sharp banding of the proteins is achieved by using a discontinuous gel system which has stacking and separating gel layers that differ in either salt concentration, pH, acrylamide concentration, or a combination of these.

The primary uses reported for SDS-PAGE are the determination of the size of a protein component, the estimation of protein purity in a solution, the purification of a protein species for other procedures, and the fractionation of a complex protein mixture prior to immunoblotting (Western blots). In general, the methods do not vary significantly from application to application, and any of several different protocols will provide acceptable results. The following section describes our conditions for SDS-PAGE and includes several tips which we have found helpful for optimizing the usefulness of this technique.

Formats and recipes

Two formats of gels are often used, minigels (7 x 10cm) and large gels (16 x 16cm). Factors which may affect the choice of format are presented in Table 1. Table 2 presents some of the gel formulations used at Promega.

Table 1. Characteristics of Minigels and Large Format Gels.

Gel Format	Sample capacity	Run times	Load volumes	General Uses
Minigels (7 x 10cm)	4µg/band, but resolution suffers	1 hour (100V until dye front reaches separating gel, then 200V)	10µl	analytical and some preparative fractionation techniques
Large gels (16 x 16cm)	20µg/band	4 hours (75V until dye front reaches separating gel, then 150V)	25µl	analytical separations and isolation of large amounts of denatured proteins

Electrophoresis/Staining of Proteins

I. Gel Preparation

(continued)

Table 2. Formulations for SDS-PAGE Resolving and Stacking Gels.
(Recipes prepare solution sufficient for two 7 x 10cm gels, 0.75-1.0mm thick.)

Resolving Gel Components	Volume for Different Percentages of Gels (in ml)				
	8%	10%	12%	15%	20%
4X lower gel buffer*	2.5	2.5	2.5	2.5	2.5
Water	5.4	4.9	4.4	3.65	2.4
40% acrylamide:bis stock	2.0	2.5	3.0	3.75	5.0
10% (w/v) ammonium persulfate	0.05	0.05	0.05	0.05	0.05
TEMED	0.005	0.005	0.005	0.005	0.005

Stacking Gel Components	Volume Added
4X upper gel buffer*	2.5ml
Water	6.6ml
40% acrylamide:bis stock	0.8ml
10% (w/v) ammonium persulfate	0.1ml
TEMED	0.01ml

*Gels using ChromaPhor Stain require a lower percentage of SDS than do other denaturing gels.

Gel Component Recipes

Acrylamide:bis stock: 37.5:1 (w/w)

For gels other than ChromaPhor-Stained, use the following:

4X lower gel buffer: 1.5M Tris-HCl, pH 8.8, 0.4% SDS
4X upper gel buffer: 0.5M Tris-HCl, pH 8.3, 0.4% SDS
Running buffer: 25mM Tris, pH 8.3, 192mM glycine, 0.1% SDS

For ChromaPhor gels, use:

ChromaPhor 4X lower gel buffer: 0.5M Tris-HCl, pH 6.8, 0.15% SDS
ChromaPhor 4X upper gel buffer: 1.5M Tris-HCl, pH 8.8, 0.15% SDS
ChromaPhor running buffer: 25mM Tris, pH 8.3, 192mM glycine, 0.035% SDS

Tips for SDS-PAGE gels

- Wear gloves; acrylamide is a neurotoxin. After gel polymerization, some unpolymerized acrylamide may be present.
- Prepare numerous gels. Wrap unused gels in a moistened paper towel and plastic wrap. Store at 4°C for up to 1 week.
- Stacking gel length should be 1cm from well bottom to top of separating gel.
- Band resolution can be improved by doubling the salt concentration in stacking and separating gels, but the gel must be run at lower voltages.
- Use fresh ammonium persulfate and high quality acrylamide. Prepare clean plates prior to mixing acrylamide and work fast.
- For sequencing applications or protein renaturation, load gel at least 4 hours post-polymerization. This allows the TEMED and ammonium persulfate to react with the gel components which decreases their chance to react with the amino-terminal end of the peptide.
- Add thioglycolic acid (0.1mM) to upper gel buffer if proteins will be subjected to sequence analysis.

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II. Sample Preparation

Sample preparation is important for obtaining accurate separation of the proteins on the basis of molecular weight. The sample treatments used should destroy any secondary or tertiary protein structure so these do not alter the migration of the proteins through the acrylamide matrix. Therefore, the sample preparation should solubilize and denature proteins, dissociate polypeptides, and reduce disulfide bonds. This is achieved through a combination of SDS, a reducing agent and heat.

Protein Sample Treatment

1. Incubate at >95°C for 2-5 minutes in a loading buffer containing SDS (1%), β-mercaptoethanol (2.5%) (or DTT) and dye.

   or

2. Heat the sample at 55°C for 15 minutes in loading buffer.

   In either case, mix the sample by vortexing before, during and after the heating step.

If the sample is too dilute for direct analysis or if salts are present which will interfere with sample resolution, concentrate the sample by TCA precipitation and resuspend in an appropriate solution.

General Precipitation Protocol (for salt removal and protein concentration)

Add TCA to 10%(w/v). Incubate 4°C for 5 minutes. Centrifuge to pellet protein. Wash with cold acetone.

III. Detection of Fractionated Protein Species

Stains

Detection of the protein species in gels is usually performed post-electrophoretically using any of a number of dyes, but may also be done during electrophoresis with certain stains, such as ChromaPhor Stain. The availability of numerous staining options makes the choice of stain difficult. This choice is dependent upon a number of parameters including speed, sensitivity, and post-staining analyses or uses. Table 3 presents information on some of these parameters for many commonly used stains.

Electrophoresis/Staining of Proteins

III. Detection of Fractionated Protein Species

(continued)

Table 3. Properties and Characteristics of Different Stains.

	Speed	Sensitivity	Elution Recovery	Applications/Comments
ChromaPhor* System				Primarily designed for use with a minigel format.
No destain	in-run	1µg	50-75%	Highly recommended for protein recovery, protein sequencing and Western blotting.
Acid enhancement (2-3 min. 5% acetic acid)	5 min.	200ng	50-75%	Can be used in 2-D gel format and for quantification with a densitometer.
Full destain (60 min. 30% methanol/ 5% acetic acid)	60 min.	60ng	not recommended	>0.035% SDS in gel components and buffers is not recommended.
				Use gels ≤1mm thick for best staining.
				Stains proteins during electrophoresis.
GhostBands Stain	15 min.	10-25ng	25-50%	Not recommended for protein recovery.
				Use ≤0.1% SDS in running buffer for proper staining.
				Band resolution is better with less complex samples.
				Negative staining of proteins which is fast and reversible.
Fast Coomassie Stain	90 min.	50ng	50-75%	Compatible with all Coomassie blue applications.
				Band intensities are slightly less than with standard Coomassie stain. Very clear background.
				Quick alternative to Coomassie blue.
Coomassie Blue	3**-18hr.	40-50ng	not recommended	Most general method used for quantifying 1- and 2-D gels with a densitometer.
Silver (3)	3-4 hr.	1-5ng	not recommended	Not recommended for quantification due to protein-to-protein variation and non-linearity of response.
				Background increases sharply if inferior quality reagents used.

* U.S. Patent No. 5,132,429 has been issued to Promega Corporation for protein staining compositions and methods.
** Microwave methods may shorten time considerably, but vapors are harmful. Hot solvents may harm oven, and gel can fracture.

Tips for staining SDS-PAGE gels

- Avoid excessive heating of gels.
- If eluting proteins from gels other than ChromaPhor gels, soak destained gels for 1-2 hours in a high concentration of SDS.

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III. Detection of Fractionated Protein Species

(continued)

Alternative detection methods

Besides staining, numerous other detection methods exist, many of which rely on the transfer of SDS-PAGE-separated proteins to a membrane, such as PVDF. Table 4 discusses some of these alternatives.

Table 4. Alternative Protein Detection Methods.

Detection Method	Sensitivity	Membrane*	Comments/Applications
Western blot	10-100pg	+	For more information, see Chapter 3.
Radioactivity	variable	+/–	Proteins labeled *in vivo* or *in vitro* (e.g., see Chapter 5), common labels ^{35}S, ^{3}H, ^{14}C, and ^{125}I. Detection limit dependent on amino acid composition and tagging method.
Ponceau S staining	100ng	+	Used to detect major protein species/MW markers on membranes before immunodetection.
India Ink	2-5ng	+	Sensitivity dependent on source and lot of ink.
Coomassie blue	30ng	+/–	Used to determine if sufficient peptide is present on membrane for sequencing (see Chapter 4).

*+ indicates use of membrane for detection; – indicates detection can be from a gel.

IV. Protein Molecular Weight Markers and Sample Molecular Weight Determination

The molecular weight of the separated proteins can be estimated by comparing the gel mobilities of a band to those generated by protein molecular weight standards. As with sizing DNA fragments, a line is plotted based on the mobilities of the standard and is a function of the log of the molecular weight. From this line, the sample molecular weight(s) can be estimated.

Table 5. Recommended Acrylamide Gel Percentages and Molecular Weight Markers.

Protein Size Range	Recommended % Acrylamide	Recommended Promega Protein Markers
40-200kDa	8%	High-Range
21-100kDa	10%	Mid-Range
10-40kDa	12%	Low-Range

Electrophoresis/Staining of Proteins

IV. Protein Molecular Weight Markers and Sample Molecular Weight Determination

(continued)

Table 6. Composition of Promega Protein Molecular Weight Markers*.

Protein	Molecular Weight	High Range	Mid Range	Low Range
Myosin	212,000	X		
β-galactosidase	116,000	X		
Phosphorylase B	97,400	X	X	
Bovine serum albumin	66,200	X	X	
Catalase	57,500	X		
Glutamate dehydrogenase	55,000		X	
Aldolase	40,000	X	X	
Carbonic anhydrase	31,000		X	X
Soybean trypsin inhibitor	21,500 (20,400/19,700)**		X	X
Horse heart myoglobin	16,900			X
Lysozyme	14,400		X	X
Myoglobin (F1)	8,100			X
Myoglobin (F2)	6,200			X
Myoglobin (F3)	2,500			X

* Promega's Protein Molecular Weight Markers are not prestained.
**A high resolution gel system described by Schägger and von Jagow (4) is used to resolve peptides in this size range. Using this system, soybean trypsin inhibitor migrates as a doublet of apparent molecular weights of 20,400 and 19,700. These values are slightly less than the soybean trypsin inhibitor apparent molecular weight obtained with the Laemmli gel system (2).

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IV. Protein Molecular Weight Markers and Sample Molecular Weight Determination

(continued)

Discrepancies in the apparent molecular weights of proteins

While many effects have been reported to cause aberrations in the apparent molecular weight of a protein, some of the effects we have seen most often are listed in Table 7.

Table 7. Aberrant Molecular Weight Determination.

Effect	Cause	Possible Solutions
Aggregation of proteins	Boiling of sample.	Treat sample at lower temperature (55°C).
	Formation of disulfide bonds between proteins in a complex mixture because of insufficient reducing agent.	Prepare new sample buffer because the reducing agent concentration has decreased.
Band streaking, high molecular weight	High salt interferes with electrophoretic mobility of proteins.	Precipitate sample and resuspend in lower salt buffer.
High molecular weight	Potassium, guanidium or divalent cations present in sample which precipitate SDS.	Precipitate sample and resuspend in different buffer.
	Proteins with >10% carbohydrate bind less SDS.	No solution.
	Hydrophilic proteins bind less SDS.	No solution.
Low molecular weight	Incomplete disulfide bond dissociation because of insufficient reducing agent.	Prepare new sample buffer because the reducing agent concentration has decreased.
	Hydrophobic proteins bind more SDS.	No solution.

V. Isolation of Proteins from Gels

Several methods for the isolation of proteins from gels are available (5-10), including the ChromaPhor Recovery System. One popular application for protein recovery from SDS-PAGE is to refold the isolated protein and study activity (see Chapter 7). Hager and Burgess (5) have described a method for this application which has been successful for some, but not all proteins.

Electrophoresis/Staining of Proteins

VI. Recording SDS-PAGE Results

Gel drying

Gels can be dried either by vacuum methods or by the use of cellulose films. Characteristics of both methods are listed below.

Vacuum methods

- fast
- gel may crack if seal is broken prematurely
- gel usually on opaque white background and can not be projected

Air-drying with cellulose film and frames

- longer to dry but little "hands on" time required
- inexpensive equipment
- gel sandwiched between clear sheets that allow overhead projection

Photography

Some suggestions for gel photography are:

Table 8. General Photographic Conditions for all Stained Protein Gels.*

Film	f/s	Exposure
Polaroid Type 665	32	1/30 second
Polaroid Type 667	32	1 second

*Gels illuminated on a light box.

Table 9. Stain-Specific Photographic Tips.

Stain	Additional Comments
ChromaPhor Stain	Use of orange filter can improve contrast. Use indirect lighting.
GhostBands Stain	Best if placed on black background. Use indirect lighting. Best method for permanent record.
Fast Coomassie Stain	Use of yellow or orange filter can improve contrast. Use indirect lighting.
Coomassie Blue	Use of yellow or orange filter can improve contrast. Use indirect lighting.
Silver	Very good contrast.

Autoradiography

If the protein samples are radioactively labeled, the results will be captured on X-ray film. First the SDS gel is dried using one of the above mentioned methods. Generally, it takes 6-15 hours to obtain an acceptable exposure of the X-ray film. The use of screens during exposure will decrease the amount of time to obtain the X-ray results. Alternatively, ^{35}S, ^{3}H and ^{14}C signal may be enhanced by fluorography. In fluorography, an organic scintillant is infused into the gel before drying. This scintillant converts the emitted energy of the isotope to visible light which increases the proportion of energy detected by the X-ray film. Using fluorography, exposure times drop to 1 to 6 hours (at –70°C). Many scintillants are available commercially.

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VIII. Ordering Information and Additional Literature Available from Promega

Ordering Information

Protein Molecular Weight Markers

Product	Size	Cat.#
Low-Range Protein Molecular Weight Markers	0.8ml	V5241
	4ml (5 x 0.8ml)	V5242
Mid-Range Protein Molecular Weight Markers	1ml	V5231
	5ml (5 x 1ml)	V5232
High-Range Protein Molecular Weight Markers	0.8ml	V5251
	4ml (5 x 0.8ml)	V5252

Stains

Product	Size	Cat.#
ChromaPhor® Protein Visualization Systems	40 minigels	V7420
	20 minigels	V7430

These systems include ChromaPhor® Stain Upper 4X Gel Buffer, Lower 4X Gel Buffer and Loading 2X Buffer.

ChromaPhor® Protein Visualization Systems with Acrylamide	20 minigels	V7260
	40 minigels	V7270

These systems include ChromaPhor® Stain, Upper 4X Gel Buffer, Lower 4X Gel Buffer, Loading 2X Buffer and Concentrated Acrylamide Solution.

Product	Size	Cat.#
ChromaPhor® Protein Recovery System	10 minigels	V7350

This system includes ChromaPhor® Stain, Upper 4X Gel Buffer, Lower 4X Gel Buffer, Loading 2X Buffer, Protein Elution Buffer, Gel Band Homogenizers, and Ultra-Free® MC Micro-concentrators. System is sufficient for isolation of 6 protein bands.

Fast Coomassie Stain	200ml	V5301

Sufficient stain for 100 minigels.

GhostBands™ Protein Detection System	20 minigels	V7360

System includes Stain, Predeveloping Solution, and Destain sufficient to stain 20 minigels and destain 6 minigels.

Gel Drying Kit (17.5 x 20cm capacity)	V7120

Include 1 pair of Gel Drying Frames, 20 sheets Gel Drying Film (25.5 x 28cm) and 12 clamps.

Gel Drying Film (25.5 x 28cm)	100 sheets	V7131

Electrophoresis/Staining of Proteins

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(continued)

Available Promega Technical Literature

Title	Lit.#
Protocols	
ChromaPhor Protein Visualization System	TB100
Gel Drying Kit	TB091
Protein Molecular Weight Markers	TB093
Ghostbands Stain	TB119
Fast Coomassie Stain	TB120
***Promega Notes* Articles**	
Laser scanning quantification of 2-D gel spots using ChromaPhor Green Stain	PN37
Improved staining of small PVDF-immobilized proteins	PN36
Selection of protein stains for SDS gel electrophoresis	PN35
ChromaPhor Protein Visualization System	PN29
Data Sheets	
Gel Drying Frames and Protein Molecular Weight Markers	DS054
ChromaPhor Protein Visualization and Recovery	DS056
Fast Coomassie and GhostBands Stains	DS081

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Immunoblotting

Basic Strategies

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Immunoblotting

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I. Standard Immunodetection Protocol

Antibodies are commonly used to detect antigens in complex mixtures. Some of the more common immunodetection methods include: ELISA (enzyme-linked immunosorbent assay) (1,2), double immunodiffusion (3,4), immunoprecipitation (5,6), immunoaffinity purification (7), and immunoblotting (8-13). This chapter will focus mainly on the immunoblotting techniques, Western blots and dot blots (14), in which the sample to be examined is immobilized on a membrane prior to detection. Another common immunoblotting technique, colony/plaque lifts, follows similar detection procedures, but will not be discussed in detail here. For more information on this procedure, see Promega's *Protocols and Applications Guide*.

Table 1. Applications of Immunoblotting Techniques.

Application	Method
Screening protein expression	
cDNA expression libraries for clone identification	colony/plaque lifts
developmental changes	dot blots/Westerns
tissue-to-tissue differences	dot blots/Westerns
in vitro translation reactions	dot blots/Westerns
Monitoring purification of proteins (screening column fractions)	dot blots/Westerns
Determining epitopes	Westerns
Screening monoclonals	dot blots/Westerns

Standard Immunodetection Protocol

Western blots, dot blots, and colony/plaque lifts all require the transfer (immobilization) of a sample onto a membrane. This is the step where the three procedures differ. With Western blots (8,9), proteins are transferred to a membrane after polyacrylamide gel electrophoresis; for dot blots (14) nondenatured antigen-containing samples are spotted directly onto a membrane using a pipet. In colony/plaque lifts, intact colonies or plaques are transferred to a membrane, then lysed to expose and bind the potential antigen.

After the transfer step, all three methods essentially follow the same basic steps to detect antigens. These steps are summarized in Figure 1.

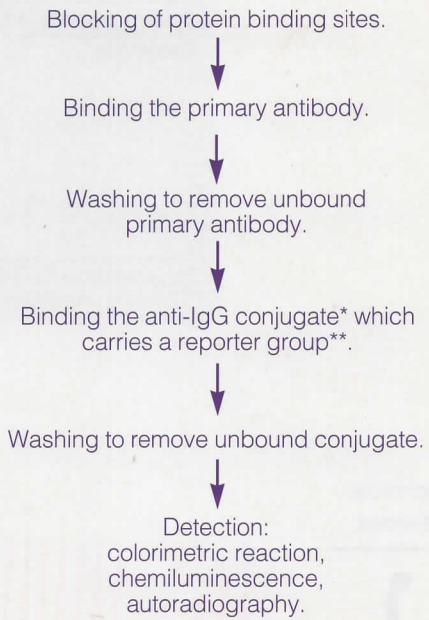
Blocking of protein binding sites.
Binding the primary antibody.
Washing to remove unbound primary antibody.
Binding the anti-IgG conjugate* which carries a reporter group**.
Washing to remove unbound conjugate.
Detection: colorimetric reaction, chemiluminescence, autoradiography.

Figure 1. Basic steps in immunodetection.

* Or other protein, such as Protein A, that recognizes common features of antibodies.

**Radioactivity or signaling enzyme

Immunoblotting

II. Sample Transfer

Detection procedures usually take approximately three hours to complete, but the time can often be dramatically reduced without a great loss in sensitivity by adjusting a number of parameters as in Promega's *Western Express™ Protocol* (supplied with the ProtoBlot® II AP Systems). The sensitivity of the assay with even a "good" antibody can often be several-fold lower on Western blots (using denaturing gels) than on "dot" blots where native proteins are spotted directly on the membrane.

Many transfer factors can affect antigen detection in subsequent steps. Some of these factors are specific to the transfer method and membrane choice, while others apply to all methods. The following section discusses some of these parameters.

Antigens can be immobilized on a number of different membranes using one of several methods. The membrane choice is dependent on many factors, including the particular antigen to be detected and the requirements of the experiment. Characteristics of some common commercially available membranes are provided in Table 2. Table 3 provides information on various transfer methods.

Tips on membranes for immunodetection

- Antigens may bind differently to different types of membranes. To maximize detection sensitivity, test different types of membranes with the particular antigen-antibody combination to be used.
- Occasionally lot-to-lot variability may be seen with some membranes.

Table 2. Characteristics of Membranes.

	PVDF*	Nitrocellulose	Nylon
Binding capacity (13)	172µg/cm²	249.1µg/cm²	149.5-175.8µg/cm²**
Comments	Requires longer blocking than nitrocellulose. Less brittle than nitrocellulose.	Often allows shorter blocking than PVDF. May not retain protein in dot blots. Good for Western blots. Can be brittle.	Requires more blocking. Does not bind protein as well as other membranes. Possibly higher backgrounds. Less brittle than nitrocellulose.

*Polyvinylidene fluoride.
**Capacity dependent on whether nylon, charge modified nylon or activated nylon.

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PPO - 2,5-Diphenyloxazole
CBBR

II. Sample Transfer

(continued)

Table 3. Transfer Methods.

Procedure	Type of Membrane	General Methods
Dot blots	nitrocellulose/PVDF	Vacuum filtration using commercial devices. Spotting 1µl volumes on moistened membranes (allow to air dry between applications if greater than 1µl used except PVDF).
Westerns	nitrocellulose/PVDF	Electrotransfer (electroelution) from acrylamide gels using commercial devices. Passive diffusion.
Colony/plaque lifts	nitrocellulose/nylon	Lay circular filter onto agar plate for 1 minute*, mark orientation, then gently pull off.**

*For some expression vector systems, longer incubation times may be required.
**If plaque lift, allow top agar to harden at 4°C for 1 hour before applying filter (12,13).

Western transfer techniques require special buffers for antigen transfer, the most common of which is Towbin Buffer (8) (see Table 4). Also Western transfers may be affected by the presence of SDS in gel and whether the gel was stained prior to transfer.

Table 4. Buffers for Western Transfers.

Buffer	Composition	Comments
Towbin buffer (8)	25mM Tris-HCl, 192mM glycine, 20% methanol, pH 8.3	
Bjerrum and Schafer-Nielson buffer (16)	48mM Tris-HCl, 39mM glycine 20% methanol, 0.0375% SDS, pH 9.2	Recommended for semi-dry transfer methods.

Tips on antigen transfer

- For Western blots, transfer efficiency can be monitored by staining the gel after the transfer.
- Efficient binding of protein to the membrane can be monitored by staining a sample lane cut from a membrane. For PVDF, use India ink (18), 0.2% Coomassie in 45% methanol, 5% acetic acid or Ponceau S. For nitrocellulose, use India ink or 0.1% amido black in 20% methanol, 10% acetic acid or Ponceau S. (Note that for either membrane Ponceau S can be used directly on the membrane with no adverse effects on subsequent detection.) Stain for 30 seconds to 1 minute. Destain in the same solution without stain.
- If the Western blot transfer efficiency is low, try longer transfer times or higher transfer voltages.
- After the transfer step, membranes can be wrapped in plastic and stored moist at 4°C.
- Once subsequent detection procedures are started, do not let the membrane dry.
- For Western blots, a method is also available for transferring gels which have already been stained, either with ChromaPhor Stain (during electrophoresis) or with Coomassie Brilliant Blue (after electrophoresis), although the detection sensitivity is slightly lower (17).
- SDS (0.1%) in the transfer buffer may improve transfer efficiency of large proteins (>100kDa).
- Methanol improves binding of smaller proteins to membranes.
- Low transfer pH (<8) or high amounts of SDS (>0.1% SDS) in the gel or buffer can lead to inefficient binding of the antigen.

Immunoblotting

II. Sample Transfer

(continued)

Table 5. Other Transfer Factors Affecting Detection.

- The amount of antigen bound per unit area of membrane.
- Some antibodies (particularly monoclonals) recognize epitopes that may become buried or denatured when the antigen is bound to surfaces such as nitrocellulose. This effect may be enhanced when blotting protein antigens from SDS-containing gels, possibly eliminating antibody recognition.
- Insufficient contact between the gel or colonies and the membrane (e.g., air bubbles).

III. Immunoblotting Solutions

Blocking, antibody incubation, and washing steps are commonly performed in buffered saline solutions. In blocking steps, the solution contains an agent to bind nonspecific binding sites present on the membrane, which lowers backgrounds. Washing solutions, in turn, remove unbound antibody and blocking agents prior to the next step. A list of common solutions and blocking agents is provided in Table 6.

General solution incubation guidelines

1. Perform at room temperature with gentle shaking.
2. Use a shallow container that is slightly larger than the membrane.
3. Solution requirements:
   Antibody: Approximately 0.1-0.15ml/cm^2 of membrane (barely submerge the membrane, protein side up.) (See Antibody Considerations section of this chapter).
   Blocking and washing: Use at least twice the antibody solution volume.

Tips on immunodetection solutions

- Elimination of Tween® 20 from buffers can lead to increased background.
- Greater than 0.05% Tween 20 in buffers may elute proteins from PVDF membranes.
- Some blocking proteins may reduce antigen recognition by certain antibodies. If this is suspected, try a different blocking protein.
- Check blocking agent for residual alkaline phosphatase activity because it could lead to high background when using an alkaline phosphatase-based detection system.
- If background is high, try a blocking solution containing 5% blot-qualified BSA in TBS or PBS.

Table 6. Common Solutions and Blocking Agents.

Solutions	
TBST	20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% Tween 20
PBST	50mM sodium phosphate, pH 7.4, 150mM NaCl, 0.05% Tween 20
Blocking Agents	
BSA, gelatin, casein	use at 1% in TBST or PBST

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IV. Antibody Considerations

Table 7. Common Polyclonal Antibody Sources. For a more detailed description, see reference 19.

Animal	Amount per injection	# injections	Amount of sera	Typical sample bleed (serum)
Mice	5-50µg	3-4	2ml	100-200µl
Rabbits	50-1000µg	3-4	200ml	10ml
Chickens	50-1000µg	3	50ml	50ml*
Rats	10-500µg	3-4	10ml	1ml

*collected from eggs

Generating antibodies

The antibody used will have a great impact on the sensitivity of a blotting system and may require that a number of individual animals be used to ensure the production of a single good source of antibody. In addition, the antibody source may determine the type of second antibody used and detection means.

A variety of approaches exists to isolate proteins for antibody production and are discussed in references 20-22. One approach, overexpression of a protein in *E. coli* and subsequent purification from, is discussed in Chapters 6 and 7. In addition, the ChromaPhor Protein Recovery System can be used to purify protein from SDS-PAGE gels for injection (23). This is particularly useful to avoid carry over of acrylamide in the sample to be injected.

The choice between monoclonal or polyclonal antibodies must also be decided. Table 8 lists the tradeoffs of these two approaches.

Table 8. Monoclonal vs. Polyclonal Antibodies.

Monoclonals	Polyclonals
Specific for epitope	Possible cross-reactivity
Possible low affinity	Large population of affinities and epitope specificities
May be difficult to produce desired antibody	Relatively fast to produce
Time-consuming to produce	Limited quantities
Quantities theoretically unlimited	Inexpensive
Expensive	Usually useful for Westerns
Possible problems with detection on Westerns	Maybe difficult to reproduce antibodies from some antigens
Can use impure antigens to produce	

Use of antibodies in immunodetection

In practice, it is useful to blot a series of known amounts of antigen by the method required for the experiment and to test several dilutions of primary antibody to maximize sensitivity and minimize background. If the primary antibody is used at too high a concentration, higher backgrounds may result. At too low a concentration, the positive signals will be weaker.

Table 9. Recommended Antibody Dilution Ranges.

Sera, ascites fluids and purified antibodies	1:200 to 1:10,000
Hybridoma tissue culture fluids (monoclonals)	1:10 to 1:100
Second antibody conjugates (Promega)	1:5,000 to 1:7,500

Immunoblotting

IV. Antibody Considerations

(continued)

Tips on antibodies

- When using a low titer antibody, increase signal by:
  - eliminating Tween 20 in buffers
  - increasing the incubations time
  - increasing the concentration of the primary antibody solution.
- Some antibodies (particularly monoclonals) recognize epitopes involving secondary or tertiary structures that are altered when the antigen is denatured or applied to membranes.
- Backgrounds will increase usually when using conjugated second antibodies at dilutions lower than 1:2,500.
- A few IgGs and other immunoglobulins (e.g., IgM) will tend to stick nonspecifically to membranes, resulting in high backgrounds.
- Localized background can result from primary antibody recognition of epitopes shared by other protein species in the sample or a primary antisera containing a mixture of antibodies with multiple specificities. The latter problem is sometimes overcome by preadsorbting the antisera to remove the cross-reacting antibodies.

Storage of antibodies

Improperly stored antibodies will lose activity over time, making detection results inconsistent. Table 10 provides some common storage guidelines.

Table 10. Antibody Storage Conditions.

Primary antisera:	–20°C in aliquots to avoid freeze/thaw cycles and prevent contamination.
Second antibodies:	4°C or in aliquots at –20°C. Avoid repeated freeze/thaw cycles.

Sodium azide (0.02%) or thimerosal (0.05%) can be added as a preservative. Azide will inhibit HRP activity.

V. Detection using AP and HRP Colorimetric Reactions/ Substrates

Detection using AP and HRP colorimetric reactions/substrates

Some researchers still use ^{125}I-labeled Protein A and autoradiography to detect the bound primary antibody. While this method is sensitive, there are methods as sensitive which avoid the hazards of radioactivity. These systems rely on the use of second antibodies which recognize common features of primary antibody and also carry a reporter enzyme. The reporter enzyme, usually alkaline phosphatase (AP) or horseradish peroxidase (HRP), catalyze reactions which generate either a colored precipitate or chemiluminescence. Colorimetric and chemiluminescence reactions provide similar levels of sensitivity (see Table 11).

Of the two common reporter enzymes conjugated to second antibodies, AP generally provides more sensitive immunodetection because of increased substrate turnover, and it is not inhibited by azide. HRP is often used when endogenous alkaline phosphatase activity or phosphate ions are present.

Table 11. Sensitivity of Various Detection Systems (24).

Methods of Detection	Dot Blot	Western Blot
^{125}I	1ng	10ng
Alkaline Phosphatase		
NBT/BCIP	5pg	25-50pg
Western Blue Substrate	5pg	25-50pg
Horseradish Peroxidase		
4-Chloro-1-Naphthol	100pg	1ng
TMB Stabilized Substrate	25pg	100pg
Chemiluminescence		
AMPPD	10pg	125pg
Luminol (ECL)	25pg	300pg

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V. Detection using AP and HRP Colorimetric Reactions/ Substrates

(continued)

Color development

The reactions catalyzed by AP or HRP produce a colored precipitate which is deposited on the membrane at the site of antibody binding. Alternatively, the reporter enzymes can catalyze chemiluminescence reactions, the results of which are captured on X-ray film.

AP-conjugated second antibodies catalyze color development using the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The solution requires mixing prior to use. New BCIP/NBT solutions (e.g., Promega's Western Blue™ Substrate for Alkaline Phosphatase) are premixed in a proprietary buffer which are ready to use and allow color development to continue for at least 24 hours for increased sensitivity (25).

Three substrates used to detect HRP-conjugated antibodies are 4-chloro-1-naphthol (CN), 3,3'-diaminobenzidine (DAB) and 3,3',5,5'-tetramethylbenzidene (TMB). The TMB Stabilized Substrate from Promega comes premixed and ready to use while CN and DAB must be prepared immediately before use. Stabilized TMB produces a stable dark blue reaction product and allows at least 3-fold more sensitive detection than CN (26). The signal is produced more quickly on a TMB blot and is longer lasting than the one produced on a CN blot.

Data recording: Photography and storage of blots

- Photograph moist membranes by placing them on top of a damp piece of filter paper on a glass plate.
- For storage, air dry the membrane on filter paper. The color will fade slightly upon drying but can be restored by moistening with water.
- Protect the membrane from light during prolonged storage.

VI. References

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Immunoblotting

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VII. Ordering Information and Additional Literature Available from Promega

Ordering Information

Immunodetection Systems*

Product	Cat.#
ProtoBlot® Western Blot AP System, Human	W3910
ProtoBlot® Western Blot AP System, Mouse	W3920
ProtoBlot® Western Blot AP System, Rabbit	W3930
ProtoBlot® II AP System with Stabilized Substrate, Human	W3940
ProtoBlot® II AP System with Stabilized Substrate, Mouse	W3950
ProtoBlot® II AP System with Stabilized Substrate, Rabbit	W3960
ProtoBlot® Western AP Blot Starter System, Human	W3970
ProtoBlot® Western AP Blot Starter System, Mouse	W3980
ProtoBlot® Western Blot AP Starter System, Rabbit	W3990
ProtoBlot® Western Blot HRP System, Human	W4080
ProtoBlot® Western Blot HRP System, Mouse	W4090
ProtoBlot® Western Blot HRP System, Rabbit	W4100
ProtoBlot® Western Blot HRP Starter System, Human	W4050
ProtoBlot® Western Blot HRP Starter System, Mouse	W4060
ProtoBlot® Western Blot HRP Starter System, Rabbit	W4070

*Each Western blotting system contains the appropriate Conjugated Second Antibody, the appropriate substrate, Tween® 20 and Blot-Qualified BSA.

ProtoBlot® Immunoscreening System	S3710

The system contains reagents necessary for plaque lifts, including two Alkaline Phosphatase Second Antibody Conjugates (anti-rabbit and anti-mouse), BCIP and NBT Development Substrates, Positive Control Phage Lysates and Positive Control Anti-β-Galactosidase Monoclonal Antibody.

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VI. Ordering Information and Additional Literature Available from Promega

(continued)

Anti-IgG Conjugates

Product	Size	Cat.#
Anti-Human IgG (H + L) AP Conjugate	100µl	S3821
Anti-Mouse IgG (H + L) AP Conjugate	100µl	S3721
Anti-Rabbit IgG (Fc) AP Conjugate	100µl	S3731
Anti-Rat IgG (H + L) AP Conjugate	100µl	S3831
Anti-Human IgG (H + L) HRP Conjugate	300µl	W4031
Anti-Mouse IgG (H + L) HRP Conjugate	300µl	W4021
Anti-Rabbit IgG (Fc) HRP Conjugate	300µl	W4011

Substrates

Product	Size	Cat.#
Western Blue™ Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
TMB Stabilized Substrate for Horseradish Peroxidase	200ml	W4121
BCIP Color Development Substrate Plus NBT Color Substrate	1.25ml/2.50ml	S3771
	6.25ml/12.50ml	S3791
4-Chloro-1-Naphthol Color Development Substrate	1g	W4111

Miscellaneous Immunodetection Products

Product	Size	Cat.#
Blot-Qualified BSA	10g	W3841
Tween® 20	2.5ml	W3831
Custom Antiserum Production		C1470

Available Promega Technical Literature

Title	Lit.#
Protocols	
ProtoBlot Western Blot AP System Manual	TM018
ProtoBlot Western Blot HRP System Manual	TM019
ProtoBlot II AP System with *Western Express*™ Protocol	TM026
ProtoBlot Immunoscreening System Manual	TM008
***Promega Notes* Articles**	
ProtoBlot Immunoscreening System	PN3
ProtoBlot Immunoblotting System	PN4
Monoclonal anti-β-galactosidase applications	PN3
Cloning of the gene for the regulatory subunit of cAMP-dependent protein kinase in *Dictyostelium*	PN3
Immunodetection using alkaline phosphatase conjugated second antibodies	PN3
Western blotting using the ProtoBlot II AP System with Stabilized Substrate	PN35
Data Sheets	
Western Blue and Stabilized TMB Substrates	DS057
ProtoBlot II AP System with *Western Express*™ Protocol	DS107
ProtoBlot Immunoscreening	DS016

Immunoblotting

Notes

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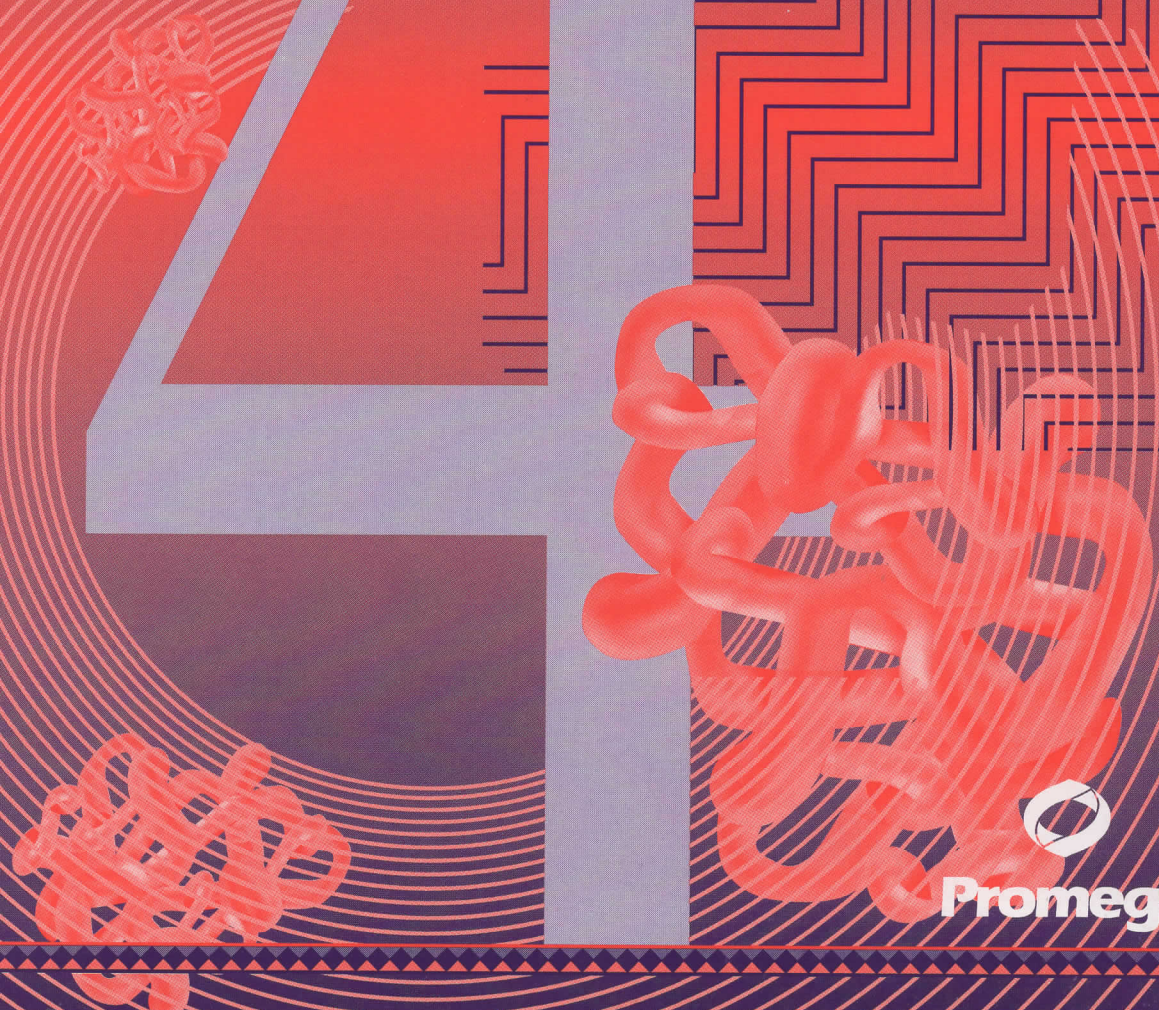
4

Protein Analysis

Basic Strategies



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Basic Strategies for Protein Analysis

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I. Partial Digests - Native Proteins

Protease or chemical digestion of proteins can answer a number of scientific questions which are difficult to answer in other ways. For example, partial proteolytic digests of native proteins have been used to remove exonuclease domains of DNA polymerases or to isolate Fab fragments from antibodies. Partial digests of denatured proteins (protein fingerprinting or Cleveland analysis) may be used to study the relatedness of two proteins with similar activities or compare epitopic or modification sites. Finally, complete digests of denatured proteins are used to obtain peptides for sequence analysis. The data obtained can be used either to generate oligonucleotide probes or locate modification sites in proteins.

Table 1 indicates the standard conditions for particular applications using chemical or proteolytic digestions, and these conditions correspond to the subsequent sections of this chapter. Also, additional information on different cleavage reagents is provided at the end of the chapter (See Tables 9-11).

Table 1. Applications and Appropriate Digest Conditions.

Application	Digest Conditions
Activity domain relationships	Partial digest, native protein
Relatedness of proteins	Partial digest, denatured protein
Location of modification	Complete digest
Sequencing (oligonucleotide probes, protein modification sites)	Complete digest or partial digest, denatured protein
Product/precursor relationships	Partial digest, denatured protein

Digest Conditions

Most native proteins are highly resistant to the action of added proteases. In general, the most susceptible regions display a high degree of flexibility and are often located between independently folding regions (domains) within the protein. However, if denaturation begins, extensive digestion of newly exposed sites can occur which may lead to a nearly complete digest.

The conditions required to maintain the native state of the protein determine the choice of protease and the fragments generated. This requirement excludes the use of several proteases based on their reaction conditions. For example, Endoproteinase Arg-C may not be useful because it requires a reducing agent, such as DTT, for activity. In addition, the target protein's specific requirements for stability must be considered. For example, a protein which requires the presence of EDTA for stability will not be easily digested with a metalloprotease which requires a divalent cation for activity.

Table 2. Suggested Starting Parameters for Partial Digests of Native Proteins.

Protein:	1µg of purified protein per digest
Starting ratios:	1:50 and 1:10 (w/w) of protease to substrate
Reaction conditions:	1 hr at stable temperature for target protein
Screen a number of proteases to determine the most suitable	

Basic Strategies for Protein Analysis

I. Partial Digests - Native Proteins

(continued)

Tips for partial digests of native proteins

- Generally, less specific proteases (e.g., Proteinase K) are recommended (see Table 11).
- For isolation of partially digested species, use proteases which can be inhibited by a covalent modification reagent (e.g., PMSF) or by removal of a cofactor. Monitor the extent of digestion by SDS-PAGE of a small sample.
- If only gel analysis of the digest is required, terminate reactions by adding TCA to a final concentration of 5%.

Analysis

The extent of digestion is determined by SDS-PAGE of inactivated reactions with detection by staining, autoradiography or immunodetection (see Chapters 2 and 3 for more information).

II. Partial Digests - Denatured Protein (Protein Fingerprinting or Cleveland Analysis)

Partial digests of denatured protein are often used to:

- determine similarity between proteins
- indicate product/precursor relationships between proteins
- examine similarities in modification sites within a protein

This technique was originally developed by Cleveland (1), and a summary is presented in Figure 1. One of the advantages of the Cleveland technique is that proteins isolated by SDS-PAGE can be substrates for digestion. Besides removing contaminating protein species, the individual subunits of a multimeric protein can be isolated to simplify analyses involving complex proteins.

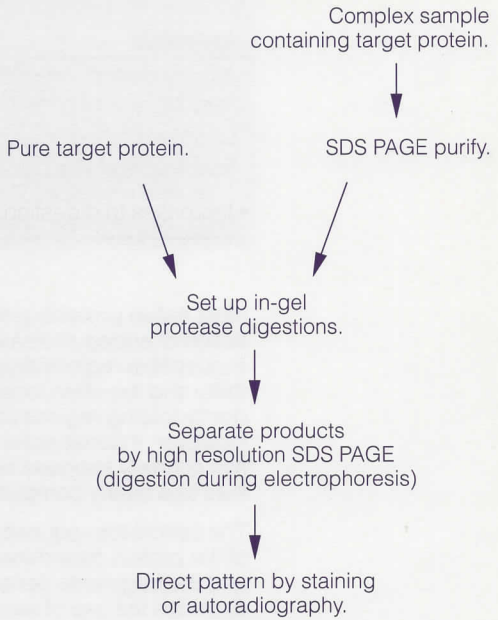
Complex sample containing target protein.
Pure target protein.
SDS PAGE purify.
Set up in-gel protease digestions.
Separate products by high resolution SDS PAGE (digestion during electrophoresis)
Direct pattern by staining or autoradiography.

Figure 1. Fingerprinting Schematic

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II. Partial Digests - Denatured Protein (Protein Fingerprinting or Cleveland Analysis)

(continued)

Digest conditions

Table 3. Recommended Starting Parameters for Partial Digests of Denatured Proteins.

Protein:	3-5µg of purified target protein per digest, either in solution or a gel slice
Starting ratio:	1:20 to 1:1000 (w/w) of protease to substrate; choose three ratios to start
Reaction conditions:	digestion occurs during electrophoresis

Tips for partial digests of denatured proteins

- Proteases must be relatively stable in the presence of SDS and reducing agents because digestion occurs during electrophoresis.
- The proteases most commonly used are Proteinase K, alkaline protease, Endoproteinase Lys-C and Endoproteinase Glu-C.
- Increases to digestion efficiency can be achieved by:
  - longer stacking gels
  - lower voltages for stacking gels
  - longer power interruption step during electrophoresis (see Table 4)
- For substrates to be isolated as gel slices, use minimum staining/destaining times to avoid permanent fixing of the sample in the gel or use the ChromaPhor Staining System (see Chapter 2).
- With gel slices, residual acetic acid from destaining procedures can cause streaking of bands in fingerprint gels.
- Gel slices for fingerprinting digests may be stored at –20°C, but better results are obtained if used immediately.

Analysis

Fingerprint gels usually require a higher concentration of acrylamide for the separation of the digestion products because of the size of the peptides generated. Table 4 presents some parameters for these gels; recipes can be found in Chapter 2 of this guide. Fingerprint products are usually detected by staining or autoradiography.

The number of peptide bands with identical mobility between two species measures the relatedness of the samples. Calvert and Gratzer (2) describe a calculation for the degree of relatedness based on this number. Additional fingerprints generated from different proteases provide further support for a finding of relatedness.

Table 4. Fingerprint Gel Guidelines.

Stacking gel:	4.5%
Resolving gel:	15% for peptides >30kDa 20% for peptides <30kDa
Thickness:	1-1.5mm to accommodate gel slice
Voltage conditions:	
Minigels:	100V, 15-20min (or until dye reaches the stacking/resolving gel interface); interrupt, 15-30min; 200V until dye reaches bottom
Large gels:	150V, 45-60min (or until dye reaches the stacking/resolving gel interface); interrupt, 15-30min; 250V until dye reaches bottom

Basic Strategies for Protein Analysis

III. Complete Digestion of Proteins

The complete digestion of proteins is routinely performed in order to obtain peptide segments that will be subjected to protein sequence analysis. The sequences obtained are used to:

- confirm the identity of a protein
- obtain amino acid sequence to generate oligonucleotide probes
- identify the site of a particular modification within a protein sequence
- identify possible epitopic sites

A number of options exist for the production and purification of peptides for sequencing, but these generally fall into two classes: 1) proteolytic digests and HPLC purification and 2) chemical cleavage and SDS-PAGE purification. Table 5 lists characteristics of these approaches.

The success of either strategy and subsequent sequencing reactions relies on:

- the amount of starting material
- the purity of the isolated peptide
- the prevention of N-terminal blockage during purification

Generally, a minimum of 100pmol of starting material is required. The purity of the isolated peptide is dependent on the resolution of the purification system chosen (HPLC or SDS-PAGE) and can be optimized by manipulation of the various parameters of each system. Numerous tricks exist to prevent blockage of N-termini, and these are provided in the SDS-PAGE purification section below.

Table 5. Complete Digests of Proteins: Chemical versus Proteolytic Digests.

	Proteolytic	Chemical
Average fragment generated	10-30 amino acids	30-300 amino acids
Purification method	HPLC	SDS-PAGE
Sequencing	Within limits for complete sequence.	Most fragments may be too large to sequence completely to point of interest.
Disadvantages	Detection based on UV absorbance, therefore what is isolated may not necessarily be a peptide.	May result in N-terminal blockage. Stain detects protein only, not other contaminants.
Advantages	Proteolytic digestion may be more specific than chemical. Improved chance to obtain sequence across desired region.	Good for combining immunodetection methods with sequence analysis. Stain detects protein only, not other contaminants.

III. Complete Digestion of Proteins

(continued)

Proteolytic digests and HPLC purification

Digest Conditions

Table 6. Recommended Starting Parameters for Complete Digests of Denatured Proteins.

Protein (minimum):	100pmol purified; 250pmol from a gel isolation*
Target ratios:	1:10-1:50(w/w) protease to substrate
Reaction conditions:	In reaction buffer containing 1M urea

*100pmol protein = protein MW(in kDa) x 0.1µg

Tips for proteolytic digests

- First, denature the substrate in 6M urea followed by dilution to 1M urea with the protease buffer.
- Overnight digestions are common. Use sequencing grade (ultrapure) proteases.
- The most commonly used proteases are Endoproteinase Glu-C, Endoproteinase Lys-C, Endoproteinase Arg-C, and Trypsin.
- If peptides generated will be used for sequencing, use sequencing grade proteases.

HPLC purification

Table 7 presents some recommended starting conditions for HPLC separation of peptides after digestion.

Table 7. Suggested Starting Parameters for Reverse Phase HPLC Isolation of Peptides.

Columns:	C8 or C18 reverse phase columns 10-25 x 0.5cm. Wash extensively with the gradient listed below to remove UV absorbing material.
Detector:	UV detector which can monitor in the 212-220nm range. Use the lowest practical absorbance range that allows the output of the detector to stay within its limits.
Gradient and components:	Use a linear gradient increasing from 100% of 0.1% trifluoroacetic acid in water to 60% of 0.1% trifluoroacetic acid in acetonitrile, 40% of 0.1% trifluoroacetic acid in water using a rate of increase in the acetonitrile component of 1% per minute. Follow this by a wash step which increases the acetonitrile component to 100%, followed by a return to 100% of 0.1% trifluoroacetic acid in water.
Flow rate:	0.4 to 1.0ml per minute for suggested columns and if compatible with the machine used.

Tips for HPLC isolations

- Identify peaks due to contaminants by running samples of reagents alone. Identify peaks resulting from digestion of the protease on itself by running a control digestion without target protein.
- Confirm the complete digestion of the target protein by SDS-PAGE analysis of a small sample of the digest.
- Determine if the HPLC has adequate sensitivity in the concentration range expected for the sample by running a digest of an inexpensive protein (e.g., BSA) in this range.
- Extensively clean the HPLC system. If HPLC is frequently used, contaminants may be a problem.
- Use only high quality HPLC reagents.

Basic Strategies for Protein Analysis

III. Complete Digestion of Proteins

(continued)

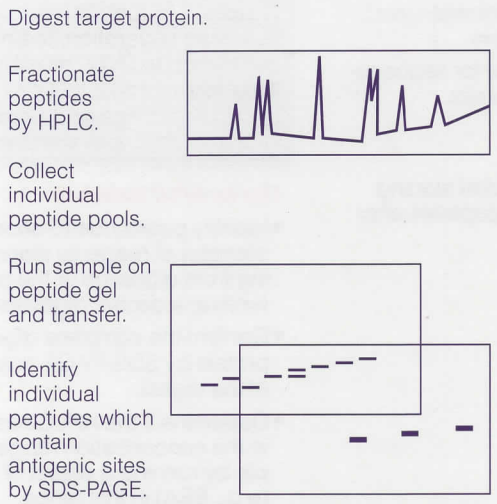
Application: Proteolytic digests and HPLC purification for epitope mapping

To sequence an epitope, a portion of the blotted complete digest is used for Western analysis (see Chapter 3) to identify the peptide binding the antibody. That band is then excised from the sample lane run in parallel and submitted for sequence analysis. Figure 2 provides an example of this procedure.

Limitations of this application

- Epitope mapping requires an antibody which recognizes the primary structure of the epitope.
- The epitope site must not contain the particular protein cleavage site.

Figure 2. Formation of peptide-specific antibody pools.


Digest target protein.
Fractionate peptides by HPLC.
Collect individual peptide pools.
Run sample on peptide gel and transfer.
Identify individual peptides which contain antigenic sites by SDS-PAGE.
Sequence antigenic peptides.

Chemical cleavage and SDS-PAGE purification

The alternative method to isolate peptides uses chemical cleavage and SDS-PAGE purification. Although these techniques are more readily available, the problem of N-terminal blockage of newly generated peptides may become more of a problem. Figure 3 presents one such common isolation scheme.

Cleavage

Table 8. Suggested Starting Parameters for Complete Chemical Digests of Denatured Proteins.

Protein (minimum):	100pmol purified; 250pmol from a gel isolation*
Cleavage reagent:	CNBr in 70% formic acid
Reaction conditions:	Room temperature for 12 hours

* 100pmol protein = protein MW(in kDa) x 0.1µg

Tips for chemical cleavage

- CNBr digestion will fail if the protein is oxidized. To ensure the protein is not oxidized, incubate in buffer containing 10mM DTT, then TCA precipitate, wash and resuspend in 70% formic acid containing CNBr.

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III. Complete Digestion of Proteins

(continued)

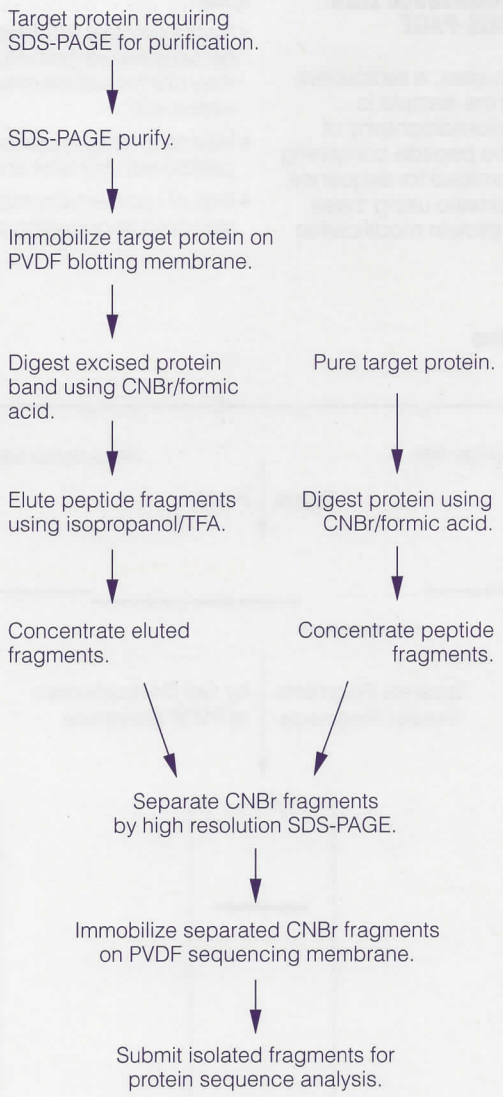

Target protein requiring SDS-PAGE for purification.
SDS-PAGE purify.
Immobilize target protein on PVDF blotting membrane.
Digest excised protein band using CNBr/formic acid.
Elute peptide fragments using isopropanol/TFA.
Concentrate eluted fragments.
Pure target protein.
Digest protein using CNBr/formic acid.
Concentrate peptide fragments.
Separate CNBr fragments by high resolution SDS-PAGE.
Immobilize separated CNBr fragments on PVDF sequencing membrane.
Submit isolated fragments for protein sequence analysis.

Figure 3. Schematic diagram of the Probe-Design Peptide Separation System procedure.

SDS-PAGE isolation methods

One very good SDS-PAGE system exists for separation of peptides generated from complete digests: the Tris-Tricine system described by Schägger and Von Jagow (3). This system operates well on peptide species from approximately 3 to 30kDa in size (approximately 30-300 amino acids in length). Most chemical cleavage methods produce peptides in this range. Proteases will generally produce fragments smaller than this, but usually one to several peptides in this range will be generated.

Tips for peptide isolation from SDS-PAGE

- Load gel at least 4 hours post-polymerization to dissipate chemical components which cause N-terminal blockage.
- Add thioglycolic acid (0.1mM), a free radical scavenger, to the cathode buffer and pre-run into the gel to reduce N-terminal blockage of separated peptides.

Transfer of proteins

Following separation, the peptide species are transferred to PVDF membranes by electroblotting techniques similar to those used for Western blotting. The membranes sold by many commercial suppliers for the sequencing of proteins are very effective in binding peptide segments and should be used as recommended by the supplier.

After the transfer, stain the membrane with Coomassie Blue to detect peptides and excise the desired fragments following recommendations of the manufacturer. These can then be given to a core facility for sequence analysis.

Tips for transfer to PVDF membranes

- Avoid methanol in the transfer buffers because methanol breakdown products may result in blocked N-termini.
- To confirm if sufficient peptide is present for sequencing, photocopy the Coomassie Blue-stained membrane. If a band is easily detected, then sufficient protein is most likely present.

Basic Strategies for Protein Analysis

III. Complete Digestion of Proteins

(continued)

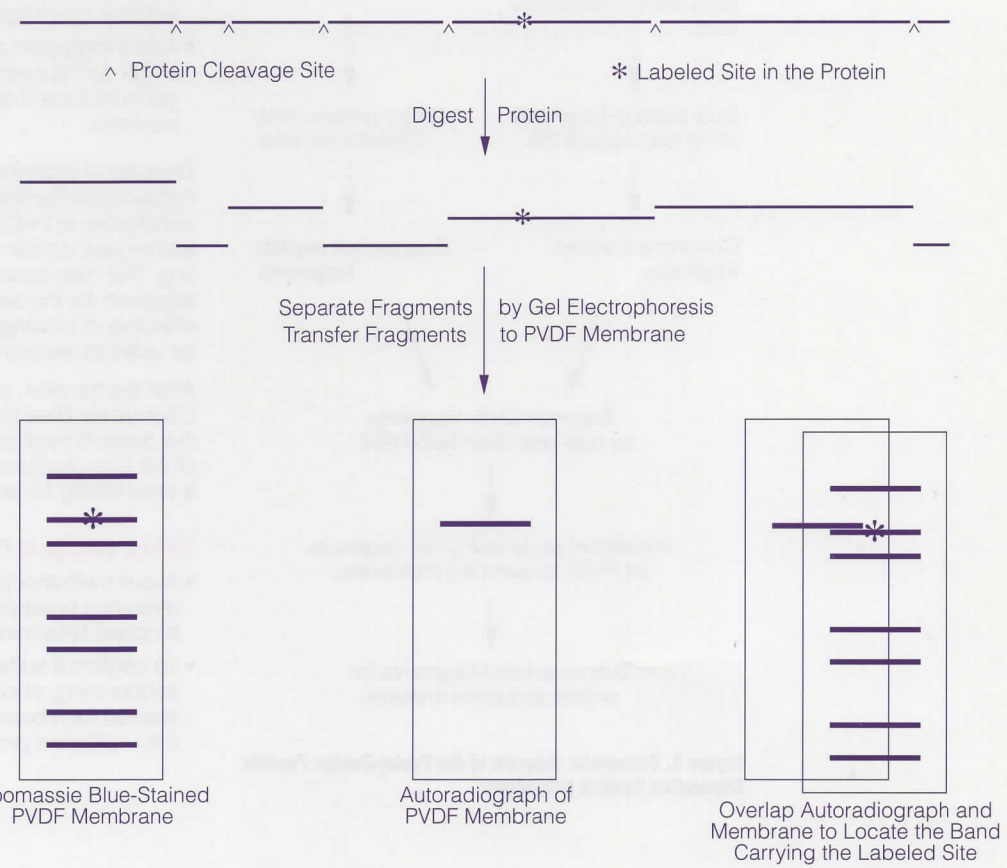
Application: Identification of modification sites using chemical cleavage and SDS-PAGE purification

For identification of modification sites, a radioactive label is used to tag the site and the sample is treated as described above. Autoradiography of the blotted samples identifies the peptide containing the modification, and this is submitted for sequence analysis. Figure 4 shows a schematic using these procedures for identification of protein modification sites.

Notes:

- Approximately 20-30 amino acid residues can be sequenced from PVDF-bound peptides. This may not be sufficient sequence to reach the modification site.
- Means to label modification must also be compatible with transfer and sequencing reactions.
- Use of radioactivity may require modifications to standard sequencing protocols (4,5).

Figure 4. Landmark mapping


^ Protein Cleavage Site
* Labeled Site in the Protein
Digest Protein
Separate Fragments
Transfer Fragments
by Gel Electrophoresis
to PVDF Membrane
Coomassie Blue-Stained PVDF Membrane
Autoradiograph of PVDF Membrane
Overlap Autoradiograph and Membrane to Locate the Band Carrying the Labeled Site

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IV. Reagent Comments

Reagent Comments

Table 9. Chemical Methods for Protein Cleavage.

Reagent	Target Sequence	Conditions
Cyanogen Bromide	Met-X	70% formic acid, room temperature, 5hrs
Partial Acid Cleavage	Asp-Pro	70% formic acid, 37°, 24-72hrs
Hydroxylamine	Asn-Gly	2M hydroxylamine, pH 9-10, 1-4hrs

Tips for chemical cleavage methods

- Works best on denatured proteins, but these conditions usually guarantee that the protein is denatured.
- CNBr digestion will fail if the protein is oxidized. To ensure the protein is not oxidized, incubate in buffer containing 10mM DTT, then TCA precipitate, wash and resuspend in a solution of CNBr in 70% formic acid.
- CNBr activity is reduced in the presence of reducing agents.

Table 10. Specific Proteases. (Enzymes available from Promega are bolded.)

Protease	Cleavage Specificity	Notes
Clostripain (Endoproteinase Arg-C)	Arg-X	Requires presence of sulfhydryl reagent (e.g., DTT) for activity.
Endoproteinase Glu-C (S. aureus V8 protease)	Glu-X (Asp-X)	Specificity dependent on digestion buffer. For cleavage at the carboxylic side of Glu, use 50mM ammonium bicarbonate, pH 7.8 or 50mM ammonium acetate, pH 4.0. For cleavage at both Glu and Asp, use 50mM sodium phosphate, pH 7.8. Inhibited by di-isopropylfluorophosphate (DFP).
Endoproteinase Lys-C	Lys-X	Works well in the presence of up to 2% SDS.
Modified Trypsin	Arg-X Lys-X	When Pro is at the carboxylic side of Arg or Lys, the peptide bond is almost completely resistant to cleavage by trypsin. Cleavage may also be considerably reduced when acidic residues are present on either side of a potentially susceptible bond (6). Inhibited by PMSF.
Pepsin	Phe-X Leu-X pairs of nonpolar residues	Acid pH denatures substrates. Neutralize to inhibit reaction.
Thermolysin	X-Leu, X-Phe, other nonpolar residues	Zn^{2+} required for activity. Tolerates 80°C. Inhibited by EDTA.
Thrombin	Arg-X	Rate of digestion dependent on amino acids in vicinity of site.

Basic Strategies for Protein Analysis

IV. Reagent Comments

(continued)

Table 11. General Proteases.

Protease	Cleavage	Notes
Alkaline Protease (Substilisin Carlsberg)	Trp-X Tyr-X Phe-X Leu-X Val-X Ile-X	Cleavage occurs at the carboxylic side of aromatic and large hydrophobic amino acids. Inhibited by PMSF.
Papain	Arg-X Lys-X Phe-X	Active in reducing agents (DTT or β-mercaptoethanol).
Proteinase K	Broad specificity	Cleavage occurs preferentially at the carboxylic sides of alipathic and aromatic amino acids.

Tips for use of proteases

- When high protease to protein ratios are used, run a control of protease only to determine bands due to self-digestion of the protease.
- Use polypropylene tubes; peptides will bind to glass.
- Avoid buffers containing potassium because a precipitate will form in the presence of SDS and may cause proteins to aggregate before electrophoresis.
- Denatured proteins are often insoluble in absence of denaturants. Disperse as finely as possible before digestion using a vortexer.

V. References

References

1. Cleveland, D.W. *et al.* (1977) *J. Biol. Chem.* **252**, 1102.
2. Calvert, R. and Gratzer, W.B. (1978) *FEBS Letters* **86**, 247.
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4. Hashimoto, Y. and Soderling, T.R. (1989) *J. Biol. Chem.* **264**, 16524.
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VI. Ordering Information and Additional Literature Available from Promega

Ordering Information

Proteases

Product	Size	Cat.#
Alkaline Protease	25mg	V5201
	125mg (5 x 25mg)	V5202
Sequencing Grade Clostripain	100µg (5 x 20µg)	V5151
	500µg (5 x 100µg)	V5152
Endoproteinase Glu-C	2mg	V5141
	10mg (5 x 2mg)	V5142
Sequencing Grade Endoproteinase Glu-C	100µg (5 x 20µg)	V5191
	500µg (25 x 20µg)	V5192
Endoproteinase Lys-C	100µg	V5211
	500µg (5 x 100µg)	V5212
Sequencing Grade Endoproteinase Lys-C	60µg (3 x 20µg)	V5131
	300µg (15 x 20µg)	V5132
Modified Trypsin	5mg	V5121
	25mg (5 x 5mg)	V5122
Sequencing Grade Modified Trypsin	100µg (5 x 20µg)	V5111
	500µg (25 x 20µg)	V5112
Proteinase K	100mg	V3021

Systems

Product	Cat.#
Protein Fingerprinting System	V6010

This system contains sufficient reagents to perform at least 20 fingerprinting reactions with each protease. Proteases provided are Alkaline Protease, Endoproteinase Glu-C and Endoproteinase Lys-C.

Product	Cat.#
Probe-Design™ Peptide Separation System	V6030

This system contains sufficient reagents to perform cyanogen bromide cleavage of approximately five target proteins as well as the reagents and membranes necessary for gel separation and immobilization of the resultant peptide fragments.

Basic Strategies for Protein Analysis

VI. Ordering Information and Additional Literature Available from Promega

(continued)

Available Promega Technical Literature

Title	Lit.#
Protocols	
Protein Fingerprinting System	TB089
Alkaline Protease	TB503
Sequencing Grade Clostripain	TB504
Endoproteinase Glu-C	TB505
Endoproteinase Lys-C	TB506
Modified Trypsin	TB512
Proteinase K	TB513
***Promega Notes* Articles**	
Protein Fingerprinting	PN24
Design of oligo probes: Preparation of proteins for sequencing with the Probe-Design Peptide Separation System	PN26
Data Sheets	
Proteases	DS039
Protein Fingerprinting	DS045
Probe-Design Peptide Separation	DS048

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In Vitro

Translation

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In Vitro Translation of Proteins

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In Vitro Translation of Proteins

I. Types of *In Vitro* Translation Systems

The primary advantage of *in vitro* translation versus *in vivo* production of protein is that *in vitro* systems allow the use of a defined template to direct protein synthesis. In this way, it is possible to have a direct correlation between a DNA sequence and protein product without the background resulting from the endogenous RNA in *in vivo* systems. *In vitro* systems also allow the specific labeling of gene products so that individual proteins can be monitored in complex reaction mixtures. In addition, *in vitro* systems are a very rapid means for protein analysis, as templates need not necessarily be cloned prior to expression.

Both eukaryotic and prokaryotic expression systems exist for *in vitro* translation. Also two basic approaches to *in vitro* translation are available: (1) programming systems with RNA templates or (2) programming systems with DNA templates (coupled transcription/translation systems). Protein production in these systems can be monitored in a number of ways. Usually, a radioactive amino acid is added to the translation reaction and after incorporation, the gene product is identified by autoradiography following SDS-PAGE (see Chapter 2). If antibodies to the protein are available, then techniques such as immunoblotting (see Chapter 3) or immunoprecipitation directly from the *in vitro* translation reaction can be used. Alternatively, if the protein has a functional activity, often this can be detected directly in the *in vitro* translation mix. If the protein was fused to a purification "tag", the protein can be isolated from the *in vitro* translation reaction and subsequently studied.

Table 2. Detection Methods for *In Vitro* Translation.

Autoradiography
Western blots
Dot blots
Immunoprecipitation
Functional assays
Affinity chromatography

Table 1. Applications of *In Vitro* Translation.

Identification of cloned DNA gene products
Immunoreactivity (e.g., epitope mapping)
Translation regulation
Protein-protein interactions
Protein-DNA interactions
Protein-RNA interactions
Production of proteins toxic to the cell
Quick screening of products generated from *in vitro* mutagenesis
Identification of signal and glycosylation sequences (with microsomal membranes)
Expression of proteins which are very protease sensitive
Translation of PCR* products (S30 Linear and TNT™ Systems only)
Protein activity studies
Test for mRNA purity prior to producing a cDNA library
Production of antigens
Production of radiolabeled protein
Molecular weight determination

*The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202, assigned to Hoffmann-La Roche. Patents pending in other countries.

In Vitro Translation of Proteins

I. Types of *In Vitro* Translation Systems

(continued)

Summary of prokaryotic and eukaryotic translation

One common mistake when using *in vitro* translation systems, particularly with cloned genes, is to not provide the system with the appropriate signals for translation (and possibly transcription). In general, prokaryotic translation requires a ribosome binding site (RBS) approximately 9 bases upstream of the start codon and no secondary structure in this region. In the case of the eukaryotic systems, sequences around the start AUG, as well as whether a message carries an $m^7G(5)ppp(5)$ cap or an internal ribosome entry site (IRES), may have a significant impact on the translatability of a gene. Figures 1 and 2 provide a summary of prokaryotic and eukaryotic translation and some of the necessary sequence elements.

Table 3. Types of *In Vitro* Translation Systems.

Translation System	Template			Promoter	RBS[1]	Signal Cleavage and Glycosylation	Approximate Protein Yield (50µl reaction)
	DNA circular	DNA linear	RNA				
Prokaryotic							
E. coli S30	yes	no	no	strong *E. coli*	yes	no[2]	100-250ng
E. coli S30 linear	yes	yes	yes	strong *E. coli* (DNA only)	yes	no[2]	100-250ng, but less efficient with RNA
Eukaryotic							
Rabbit Reticulocyte	no	no	yes	no	no	yes	50-200ng
Wheat Germ	no	no	yes	no	no	yes with modification[3]	30-150ng
TNT Rabbit Reticulocyte	yes	yes, but less efficient	no	SP6, T3 or T7[4]	no	yes	150-300ng
TNT Wheat Germ	yes	yes	no	SP6, T3 or T7[4]	no	no	100-200ng

[1]RBS = ribosome binding site.

[2]Some bacterial processing has been observed with the β-lactamase gene.

[3]In order for the protein to become associated with the vesicle and processing to occur, a signal recognition particle (SRP) must interact with the protein. During the preparation of the wheat germ extract, the SRP is removed in an EDTA step. In order for this process to be optimized when using the wheat germ, either exogenous SRP must be added, or the membranes should be prepared excluding the EDTA stripping step. Included is a reference for the use on microsomal membranes in conjunction with wheat germ extract (15).

[4]Promoter choice may have a significant impact on translation in these systems. Please refer to this section for more detail.

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I. Types of *In Vitro* Translation Systems

(continued)

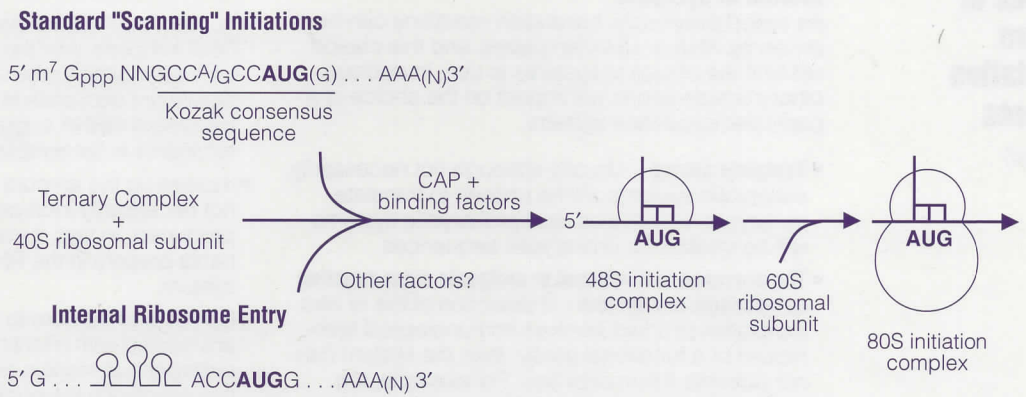

Figure 1. Generalized schematic of eukaryotic translation initiation.

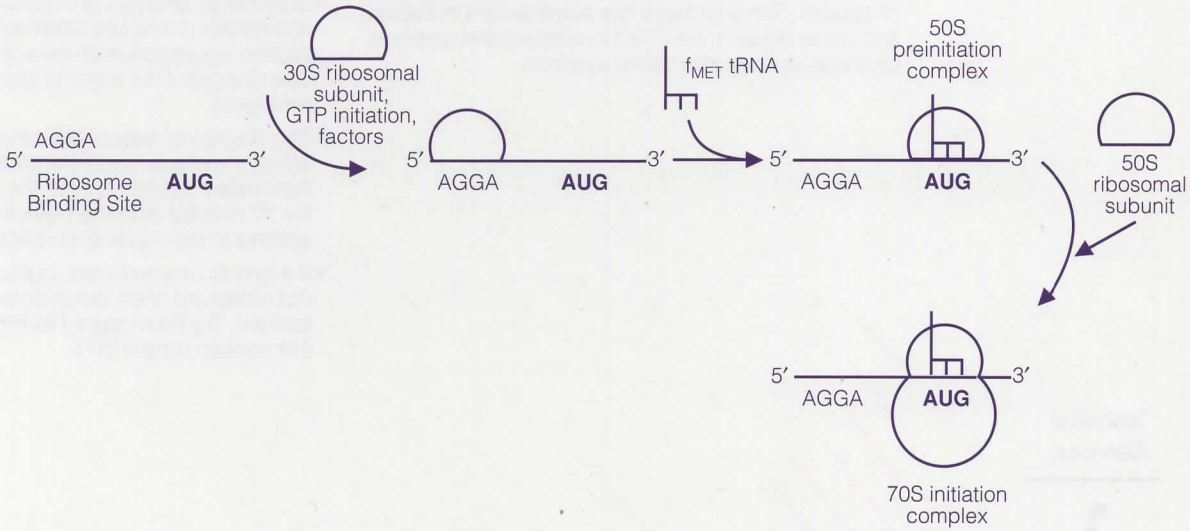

Figure 2. Generalized schematic of prokaryotic translation initiation.

In Vitro Translation of Proteins

I. Types of *In Vitro* Translation Systems

(continued)

Choice of systems

As stated previously, translation reactions can be driven by RNA or DNA templates, and this choice will limit the choice of systems to use. In addition, other considerations will impact on the choice of a particular translation system:

- **Template source** – Usually although not necessarily, eukaryotic systems will be chosen to translate eukaryotic sequences, and prokaryotic systems will be chosen for prokaryotic sequences.
- **The presence of functional or antigenic cross-reacting components in a system** – If detection of the *in vitro* translation product involves immunological techniques or a functional assay, then the system cannot possess these activities. For example, the presence of globin in a rabbit reticulocyte lysate dictates the use of wheat germ extracts for studies of the globin gene.
- **Eukaryotic post-translational modification or processing** – Microsomal membranes, which provide the necessary components for these processes, work most efficiently in rabbit reticulocyte lysates.

A number of other factors will determine the choice of system. Some of these are summarized in Table 3, and more detail is provided in subsequent sections on these various alternative systems.

Tips for in vitro *translation reactions*

- To test for inhibitory components in the RNA or DNA template, mix the experimental template with a template known to be translated well. A significant decrease in the expression of the well translated mRNA suggests the presence of contaminants in the template.
- Increasing the amount of RNA/DNA added does not necessarily increase the amount of protein produced. In fact, it may increase any contaminants present in the RNA/DNA and inhibit the system.
- Do not add calcium to the reaction. All extracts are treated with micrococcal nuclease to remove endogenous nucleic acids. The presence of Ca^{2+} can reactivate the nuclease, destroying the added template.
- Usually, translations are performed at approximately 30°C. However, in some cases, it may be advantageous to lower the temperature (25-29°C). For example, if synthesizing a heat-labile protein (e.g., luciferase), a lower incubation temperature can possibly increase total protein yield, as well as yield of functional activity.
- SDS-PAGE analysis of *in vitro* translation products sometimes produces aberrant results because of protein aggregation or incomplete denaturation. See Chapter 2 for ways to circumvent these problems.
- The degree of secondary structure in an RNA may affect the efficiency of translation. To decrease secondary structure, heat the mRNA at 65-70°C for 10 minutes and then quick cool on ice prior to adding to the translation reaction.
- If a protein is produced, but functional activity is not observed, then disulfide bridges may not have formed. Try Promega's Flexi™ Lysate which does not contain added DTT.

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II. Rabbit Reticulocyte Lysate Translation Systems

Rabbit reticulocyte lysate (1) and wheat germ extracts (2) are eukaryotic translation systems used to identify gene products from mRNA isolated *in vivo* or from mRNA transcribed *in vitro* by a phage polymerase (e.g., T7, T3 or SP6). Coupled transcription/translation systems (Promega's TNT Systems) also exist for both rabbit reticulocyte lysates and wheat germ extract (3-5) and will be discussed later (see "Eukaryotic Coupled Transcription/Translation Systems" section this chapter).

Sequence and construct considerations

The presence of certain nucleic acid sequence elements can have a profound effect on initiation fidelity and translation efficiency. In fact, many times these elements can be the source of erroneous results if not considered. Table 4 lists some of these elements and their effects in translation systems.

Table 4. Sequence Elements Affecting Translation in Rabbit Reticulocyte Lysate Systems.
Please also see Table 7 below for salt effects on these elements.

Sequence element	Comments
Start codon context	The "Kozak" consensus sequence acts strongly in rabbit reticulocyte lysate (6). This sequence has a purine in the –3 position and a G in the +4 position.
Poly(A) tail (or 3´ untranslated region)	The presence of a poly(A) tail at the 3´ end of a coding region can increase translation 7-fold. However, translation can be inhibited directly by adding just polyA, or by adding increasing amounts of a polyadenylated message.
Presence of 5´ cap structure	Some RNAs translate more efficiently if they contain a 5´mG cap. Internal (or false starts) are more often seen with uncapped messages. Uncapped messages translate more efficiently if they contain internal ribosome entry sites (IRES), such as those found in picornovirus mRNAs.
5´ untranslated region	If the 5´ upstream region of the EMCV mRNA (containing an IRES) replaces the upstream sequence of the experimental mRNA, we observe a 16-fold increase in translated product provided a high concentration of KCl (110-120mM; not potassium acetate) is added to the reaction. There are other such "enhancing" 5´ UTRs (such as from β-globin) which also can be placed upstream of the mRNA of interest to increase translation product. IRES has an adverse effect on translation in wheat germ extracts.

In Vitro Translation of Proteins

II. Rabbit Reticulocyte Lysate Translation Systems

(continued)

Tips on structural features affecting rabbit reticulocyte lysate translation

- If smaller, secondary bands appear after analysis of a translation reaction by SDS-PAGE, internal start sites may be present in the sequence. To test this possibility, translate a truncated gene sequence. If present, the proposed internal start product should be truncated by the same amount.
- A way to reduce the amount of internal initiation from a specific message is to use the proper K^+ concentration and use KCl rather than potassium acetate (see below).

Template preparation

The preparation of the RNA template used in rabbit reticulocyte translations can affect the efficiency of translations or may result in other abnormalities. Either *in vivo* RNA or RNA produced *in vitro* by phage polymerases, such as SP6, can be used as templates in reticulocyte translations. The optimal concentration of template varies, however, with the isolation method and the individual RNA. Table 5 provides some key guidelines for these preparations, and Table 6 provides information on various additional purification methods.

Table 5. RNA Template Guidelines for Rabbit Reticulocyte Translations.

Type	Concentration	Comments
In vivo RNAs		
Total RNA	100-200µg/ml	Translates inefficiently because of the abundance of rRNAs and tRNAs in the preparation.
Poly(A)+ RNA	5-20µg/ml	Poly(A)+ fractionation removes the tRNAs and rRNAs, increasing translation efficiency. Poly(A)+ RNA isolated from plants may contain polysaccharides which inhibit reticulocyte translations. If >2µg/50µl reaction is used, an increase in internal translation starts may be observed.
In vitro RNAs		
Tris-based system (e.g., Promega's Riboprobe® System)	10-20µg/ml	For either method, more consistent results are obtained if the RNA has been purified (see Table 6). Capping of *in vitro* messages with a 5´ dinucleotide may increase the translational efficiency.
HEPES-based system (e.g., Promega's RiboMAX™ System)	10-80µg/ml	Larger quantities of RNA may be added to translations if it was prepared by the HEPES-based system. This may eliminate the need to cap *in vitro* RNAs.

Table 6. General Procedures for Purifying Template RNAs.*

Organic extractions followed by ethanol precipitation	Extract with phenol:chloroform:isoamyl alcohol, followed by a chloroform:isoamyl:alcohol extraction. Then precipitate with 0.3M sodium acetate and 2.5 volumes ethanol. Completely remove any traces of chloroform.
Chromatography	Sephadex G-25 or G-50, RNase-free. Autoclaving material can help reduce, but may not eliminate RNase contamination.
LiCl precipitation	Not generally recommended because it excludes small molecular weight RNAs. Also requires further ethanol precipitation to remove inhibitory Cl^- ions.

*For poly(A)+ RNA isolations, Promega's RNAgents® Total RNA Isolation Systems followed by PolyATtract® System for poly(A)+ purification has been used successfully. Alternatively, Promega's PolyATtract System 1000 can be used.

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II. Rabbit Reticulocyte Lysate Translation Systems

(continued)

Tips for template preparation for use in reticulocyte lysates

- Polysaccharides, double-stranded RNA and oxidized thiols are inhibitors of reticulocyte translations (7).
- A 5-fold reduction of rGTP and a corresponding increase in 7mG(5')ppp(5')G cap analog in *in vitro* transcription reactions will produce two-thirds capped products, but the yield of the transcription reaction drops (20-50% of standard reaction). The ratio of cap analog:rGTP in the reaction can be varied from 10:1 to 1:1 to balance the percentage of capped products and the efficiency of the transcription reaction.
- RNA produced *in vitro* can be added directly to translation reactions. In this case, dilute 1:10 to 1:50 in the reaction.
- RNA generated *in vitro* may contain additional truncated products from premature termination. These truncated RNAs could then produce truncated proteins in the translation reaction.
- *In vitro* transcriptions using a linearized template having a 3' overhang may produce large mRNAs including anti-sense mRNA. These RNAs may produce aberrant translation products or inhibit translation of the desired product. To avoid this problem, treat the 3' overhang with Klenow DNA polymerase prior to the transcription reaction (8).

Rabbit reticulocyte reaction parameters

As with all *in vitro* translation systems, the optimal salt conditions vary for individual templates and should be determined empirically. The optimal salt concentrations can increase translation as much as 10-fold. The salts having the greatest effects are Mg^{2+} and K^+.

- **Mg^{2+}** – The concentration of Mg^{2+} is critical for translation, and the titration curve is very steep. Typically, the Mg^{2+} concentration in a translation reaction should be 2mM +/– 0.5mM.
- **K^+** – Either potassium acetate or KCl can be added, but recent evidence suggests that while the titration curves with KCl are steeper, the fidelity of the translation initiation is greater than with potassium acetate, especially with uncapped mRNA. The addition of certain untranslated regions can affect the K^+ optimum and some generalized rules of K^+ levels can be found in Table 7.

Table 7. Guidelines for KCl Concentration in Rabbit Reticulocyte *In Vitro* Translation.

Template sequence composition	Suggested KCl concentration
In vitro transcript	40-70mM
Poly(A)+ containing transcript (uncapped)	60-90mM
IRES containing transcript (uncapped)	100-120mM
Capped RNAs	70-100mM

Other components can be added to rabbit reticulocyte translation reactions to increase translation fidelity and efficiency. These are summarized in Table 8.

In Vitro Translation of Proteins

II. Rabbit Reticulocyte Lysate Translation Systems

(continued)

Table 8. Other Additives to Enhance Rabbit Reticulocyte *In Vitro* Translations.

Additive	Concentration	Comment
3', 5'-cAMP	1-10mM	May stimulate translation for a short period.
GTP	2mM	Helps prevent activation of inhibitory kinases of eIF2α.
Glucose-6-phosphate	0.5mM	In some cases, stimulates translation.
Polyamines (e.g., spermidine, diamines)	0.1-1.5mM (spermidine) 0.1-4mM (diamines)	The addition of spermidine (approximately 0.4mM) will affect the level of Mg^{2+} in reticulocyte lysates by dropping the optimal Mg^{2+} concentration needed for translation (9).
tRNAs		The tRNA population of the reticulocyte cell is optimized for expressing globin. If the gene of interest contains codons to which the cognate tRNAs are rare in reticulocyte lysate, translation could be limited. Supplement with calf liver tRNAs. If the gene of interest contains an abnormally high concentration of a particular amino acid or codon or utilizes a different genetic code (e.g., mitochondrial genes and some lower eukaryotes), it may be necessary to purify the tRNA from the same source of the mRNA.
RNasin® Ribonuclease Inhibitor	40 units/50µl reaction	Enhances the synthesis of full-length proteins by preventing RNA degradation (10).

In some experiments, especially when a defined template (e.g., an *in vitro* transcript) is used, autoradiography is used to monitor protein production. For reticulocyte translations ^{35}S-methionine is most frequently used since the pool size is low in reticulocytes (5µM) and methionine is available at high specific activity. Use only "translation grade" ^{35}S-met (such as Amersham Cat.# SJ1515 or SJ1015) otherwise binding may occur to a 42kDa reticulocyte protein. Leucine and cysteine are also commonly used (pool sizes of 2-5µM) (11).

Tips for reticulocyte lysate reaction

- Increasing the amount of labeled amino acid (by as much as 2-fold) can usually increase incorporation of the label into the protein, decreasing exposure times required.
- For some low expressing messages, it may be worthwhile to optimize K^+ and Mg^{2+} using a system, such as Promega's Flexi Lysate, before trying wheat germ extracts.
- There are easy methods to assay for Mg^{2+} concentration, such as the Lancer kit, which we use to determine the Mg^{2+} concentration of our Flexi™ Lysate System.
- If the protein has an abnormally high percentage of Met or Cys residues, chasing the translation reaction with 20µM Met or Cys may prevent premature termination of translation.
- If the translated protein contains a large number of the amino acid used for labeling (e.g., methionine) and the amount of label is used up too quickly, premature termination may result, producing secondary bands upon analysis.

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II. Rabbit Reticulocyte Lysate Translation Systems

(continued)

- ^{35}S-labeled amino acids are easily oxidized to translation-inhibiting sulphoxides and should be stored in aliquots with buffer containing DTT or β-mercaptoethanol at –70°C.
- If the micrococcal nuclease treatment was incomplete, a globin band is apparent at 10kDa.
- Post-translational modifications, such as isoprenylation or myristylation, may occur in reticulocyte translations and can affect the rate of migration of the translated product in the SDS-PAGE. Because these processes may only affect a percentage of the molecules, two or more bands may be apparent on SDS-PAGE.
- Because of the large amount of globin in reticulocyte lysates, proteins of approximately the same size (approximately 10kDa) may migrate abnormally.

Eukaryotic processing: Microsomal membranes

When appropriate, translation products may be further analyzed for eukaryotic co-translational processing and core glycosylation by the addition of canine pancreatic microsomal membranes to a standard translation reaction (12). Processing events are generally detected as shifts in the apparent molecular weight of translation products. Generally, this technique should only be used in conjunction with rabbit reticulocyte lysate-based translation reactions.

Tip for use of microsomal membranes

- In general, increasing the amount of membranes in the reaction increases the proportion of polypeptides translocated into vesicles but reduces the total number of polypeptides synthesized.

III. Wheat Germ Extracts

Wheat germ extracts provide an alternative to rabbit reticulocyte lysate and are generally used when translation is inefficient in reticulocyte lysates (2). Wheat germ extract readily translates certain RNA preparations, such as those containing low concentrations of double-stranded RNA (dsRNA) or oxidized thiols, which are inhibitory to reticulcyte lysate. In addition, it is the system chosen when cross-reacting (or cross-functional) components are present in reticulocyte lysates and antibody detection (or functional assays) are used.

Sequence and construct considerations

As with reticulocyte lysates, the context around the start codon greatly influences translation in wheat germ and poly(A) sequences at the 3' end of a coding region enhance translations. For more information on these and other structural features affecting translation in wheat germ extract, please refer to Table 4.

Template preparation

As with all *in vitro* translations, poor quality template can greatly affect the outcome of the reaction either by decreasing translation efficiency or resulting in other abnormalities. Templates, either *in vivo* RNAs or RNA from *in vitro* transcription reactions, should be prepared as for rabbit reticulocyte translations (see Table 6). The template concentration required in the reaction varies with the individual RNA and its preparation, and should be determined empirically. Table 9 provides some starting guidelines for various RNAs.

In Vitro Translation of Proteins

III. Wheat Germ Extracts

(continued)

Table 9. RNA Template Guidelines for Wheat Germ Extract Translations.

Type		Concentration	Comments
In vivo RNAs	Total RNA	100-200µg/ml	Translates inefficiently because of the abundance of rRNAs and tRNAs in the preparation.
	Poly(A)+RNA	5-80µg/ml	Poly(A)+ fractionation removes the tRNAs and rRNAs, increasing translation efficiency. If >2µg/50µl reaction is used, an increase in internal translation starts may be observed.
In vitro RNAs		4-10µg/ml	More consistent results are obtained if the RNA has been purified (see Table 6). Capping of *in vitro* messages with a 5' dinucleotide may increase the translational efficiency.

Also read the *Tips for template preparation* in the "Rabbit Reticulocyte Lystate Translation" section, pg. 41.

Reaction parameters

The optimal conditions for translation in wheat germ extract will vary with the individual RNA and should be determined empirically. Table 10 provides information on some of these variables. In addition, translation in wheat germ extracts is inhibited by Na^+, NH_4^+, Cl^- and amino acid analogs (10).

Eukaryotic processing: Microsomal Membranes

Microsomal vesicles are used to study co-translational and post-translational processing of proteins. In order for the protein to become associated with the vesicle, a signal recognition particle (SRP) must interact with the nascent polypeptide. During the preparation of the wheat germ extracts, the SRP is removed, and this is apparently the reason why processing reactions using microsomal membranes are more efficient in reticulocyte lysates (15).

In order for this process to be optimized when using wheat germ, either exogenous SRP must be added, or the membranes should be prepared excluding the EDTA stripping step. Reference 15 provides information for the use of microsomal membranes in conjunction with wheat germ extracts.

Table 10. Some Variables Affecting Translation in Wheat Germ Extract.

Additive	Concentration	Comments
potassium acetate	75-120mM (13)	
KCl	30-80mM (13)	
$MgCl_2$	2.0-3.5mM (13)	
spermidine	0.6-1.2mM	Increases synthesis of large proteins (14).
RNasin® Ribonuclease Inhibitor	40 units/50µl reaction	Enhances the synthesis of full-length proteins by preventing RNA degradation (10).

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IV. Eukaryotic Coupled Transcription/ Translation Systems (TNT Systems)*

The TNT Coupled Transcription/Translation Systems offer a fast and efficient alternative for eukaryotic *in vitro* translation of genes cloned downstream of SP6, T7 or T3 RNA polymerase promoters (3-5,16,17). These systems produce protein starting with a DNA template, eliminating the time required to perform *in vitro* transcriptions. In addition to the time savings, coupled transcription/ translation reactions generally produce 2- to 6-fold more protein than standard transcription and translation reactions.

Sequence and construct considerations

The sequence context around the AUG start plays an important role in the TNT Systems as they do in standard reticulocyte lysates and wheat germ (see above). However, the phage polymerase promoter chosen to drive the transcription portion of the reaction also impacts on the efficiency of translation in the TNT Systems. Table 11 summarizes the promoter effects, and Table 12 describes other sequence effects.

Table 11. Phage Promoter Effects in TNT Systems.

	Promoter		
System	**SP6**	**T7**	**T3**
TNT Rabbit Reticulocyte Lysate	Circular DNA provides the most efficient translation. Use of linearized constructs under the SP6 promoter is not recommended.	Circular DNA is the most efficient, but linear works acceptably.	Circular DNA is the most efficient, but linear works acceptably.
TNT Wheat Germ Extract	Circular DNA provides the most efficient translation. Linear DNA is not recommended.	Linear DNA must be used unless a T7 termination sequence is located downstream of the coding region.	Circular or linear DNA can be used for efficient translation.

Table 12. Sequence Elements Affecting TNT Translations.

Sequence element	Comments
Start codon context	The "Kozak" consensus sequence acts strongly in rabbit reticulocyte (6). This sequence has a purine in the –3 position and a G in the +4 position.
Poly(A) tail (or 3' untranslated region)	The presence of a poly(A) tail at the 3' end of the gene have been observed to enhance translation.
Presence of 5' cap analog	Addition of $m^7G(5)ppp(5)G$ cap analog inhibits translation in TNT Systems because the free analog binds translation initiation factors.
5' untranslated region	If a 5' region carrying an internal ribosome entry site (IRES), such as that in the EMCV mRNA, is cloned upstream of the coding sequence and an additional 40-60mM KCl is added, then translation efficiency may be enhanced. However this does not work in the wheat germ-based systems.

*Patent pending

In Vitro Translation of Proteins

IV. Eukaryotic Coupled Transcription/ Translation Systems (TNT Systems)

(continued)

➤ ***Tips on structural features affecting TNT translation***

- If smaller, secondary bands appear after analysis of a translation reaction by SDS-PAGE, internal start sites may be present in the sequence. To test this possibility, translate a truncated gene sequence. If present, the proposed internal start product should be truncated by the same amount.
- T7 terminator sequence: 5' ATAACCCCTTGGGG CCTCTAAACGGGTCTTGAGGGGTTTTTTG 3' (18).

Template preparation

The TNT Systems only use DNA as a template. DNA templates obtained from standard alkaline lysis miniprep procedures or Magic™ DNA Purification Systems will often work in these reactions. If the template is linearized, then ethanol precipitate it before using in a TNT coupled transcription/translation reaction. Optimal results are obtained with 0.2-2µg DNA per reaction; adding more DNA does not necessarily increase the amount of protein produced.

TNT reaction parameters

Because of the nature of this coupled system, it is not recommended to adjust salt concentrations to enhance translation of a sequence as can be done with rabbit reticulocyte lysates or wheat germ. An exception to this rule concerns IRES containing constructs, which can be enhanced by the addition of 40-60mM KCl (not potassium acetate).

Optimal translation is obtained in 60-120 minutes (30°C) for T7 and T3 promoters. For SP6-driven constructs, translation can continue for up to 120 minutes.

Eukaryotic processing: Microsomal membranes

Microsomal membranes have been successfully used with the TNT Rabbit Reticulocyte Systems (16). Gene expression using microsomal membranes, however, will decrease approximately 50-80% over levels observed without this addition, which is typical for processing in any translation system. Even with the decrease in production, sufficient product is produced to be detected by autoradiographic techniques. Microsomal membranes, when used in TNT Wheat Germ Systems, dramatically decreases protein expression and are not recommended to study processing in these systems.

V. S30 Systems

Prokaryotic systems

The bacterial coupled transcription/translation systems (designated S30 for supernatant-30,000g) have been used for many years to examine translation products and study protein-DNA and protein-protein interactions. Two types of S30 systems are available (19-21). The standard system allows the expression of coding sequences in supercoiled plasmids if under the control of an *E. coli* promoter. Linear DNA templates are degraded in these systems. The second type of S30 system is generated from an *E. coli* strain which has an inactivated exonuclease V gene. This mutation allows linear or plasmid DNA to be transcribed and subsequently translated. RNA may also be used, but translation is less efficient. In addition, S30 extracts prepared from *E. coli* strains deficient in *ompT* endoproteinase and *lon* protease activity result in greater stability of newly synthesized proteins.

Sequence and construct considerations

The primary requirement for the examination of a gene product in an S30 system is a DNA template containing the gene under control of a bacterial promoter with a suitable ribosome binding site. Some commonly used promoters for good expression are *lac*UV5, *tac*, and λP_L. Phage promoters, such as T7, can be used if the appropriate RNA polymerase is added to the reaction, but yields may be lower. Sequences of some promoters which have been used are shown in Table 13.

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V. S30 Systems

(continued)

Table 13. *E. coli* Consensus Promoter Sequences.

-35*	-10*
TTGACA	TATAAT

* Sequences are approximately the designated number of bases upstream of the start codon.
**T7, T3 and SP6 phage promoters may be used in S30 extracts, but their use requires the addition of these phage polymerases to the S30 mix.

A ribosome binding site appropriately spaced from the initiation codon is also required for efficient translation initiation. No absolute requirement exists for one particular sequence and translation efficiencies *in vitro* seem to mimic those *in vivo*. Examples of efficient *E. coli* upstream translation initiation sequences are shown in Table 14.

Table 14. Ribosome Binding Site from pGEMEX® Vector.

5'TTAACTTTAAGAAGGAGATATACATATG3'
Shine-Dalgarno (22) Met

Tips for structural features affecting in S30 systems

- It is important to remember when using a promoter in the S30 system that whatever repressors or activators affect the promoter *in vivo* may influence the expression from that promoter *in vitro*. For example, the wild type *lac* promoter will require CRP and cAMP for activation. Promega's S30 Premix contains cAMP and IPTG to allow expression from *lac* and *tac* promoters.
- Promoters are ideally placed 40 bases upstream from the gene, but usually work at greater distances as well.
- When in doubt about the promoter to use, the *tac* or λP_L promoter are usually good choices.
- Some bacterial promoters, such as the wild type *lac* promoter, show reduced transcriptional activity when the DNA is not superhelical.

Template preparation

DNA quality is the single most important factor affecting translation in S30 systems because the S30 systems are extremely sensitive to contaminants. Common contaminants of DNA including RNA, SDS, excess Na^+, Mg^{2+} and K^+ salts, and ethanol can greatly reduce activity.

In general, supercoiled DNA works better than linear and should be of the highest quality. CsCl-purified DNA produces the best results. The amount of DNA required will depend somewhat on the template, but usually 1-5µg per reaction will be sufficient.

Linear DNA templates are particularly useful for generating and characterizing protein fragments. Since the added template defines the protein produced, simple fragmentation of the template at a defined site such as a restriction site will yield a protein fragment of the corresponding size. These protein fragments can be used for structure/function analysis as well as epitope mapping. Using a similar approach, PCR can be used to generate templates which contain a promoter, ribosome binding site, the desired coding region, and a translation termination signal. Strategic placement of the upstream and downstream primers allows for the precise deletion of both upstream and downstream coding regions. This process does not require the cloning of the PCR-generated product as the template can be used directly. Figure 3 describes an application of this use for epitope mapping which can also be applied to TNT Systems (see "Eukaryotic Coupled Transcription/Translation" section of this chapter).

In Vitro Translation of Proteins

V. S30 Systems

(continued)

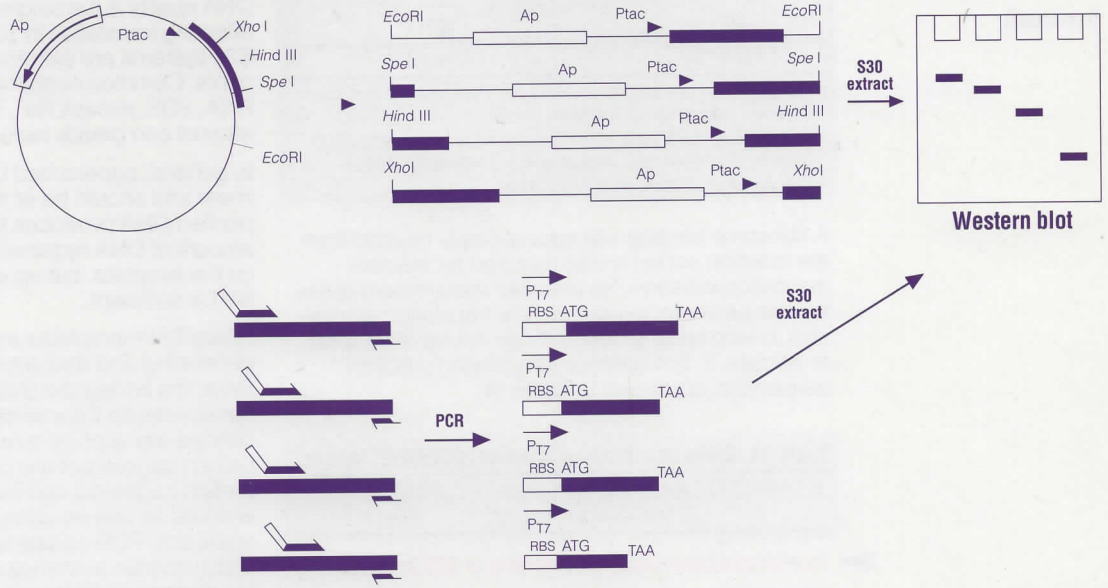

Figure 3. Epitope mapping using translation of restriction digests or PCR fragments in S30 linear system.

Tips for template preparation for S30 Systems

- Use sodium acetate for ethanol precipitations. Ammonium ions can reduce template activity if present in concentrations greater than 50mM.
- Increasing the amount of DNA added to the reaction may not necessarily produce more protein. In fact, excess DNA can inhibit the reaction and may produce translational products from false start sites.

S30 reaction parameters

S30 extracts exist which can utilize either supercoiled circular or linear DNA templates. The two major advantages to using a linear-competent S30 system are the ability to do C-terminal deletions using restriction enzymes on plasmids and the ability to assay PCR products directly to generate both N- and C-terminal deletions.

Various reaction parameters will have to be empirically determined for optimal translation of a particular RNA. Proteins are rarely synthesized at a level above 1-2µg per reaction; typical yields are from 50-250ng per reaction. Table 15 provides some parameters affecting translation in S30 systems and approximate starting guidelines.

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V. S30 Systems

(continued)

Table 15. Reaction Parameters Affecting S30 Translation.

Parameter	Allowable Additional Concentration	Comment
Mg^{2+}	0-2mM	The most critical parameter in S30 Systems.
K^+	0-100mM	
NH_4^+	0-10mM	S30 translations will be inhibited above 50mM NH_4^+.
glycerol	<2%	Above 2% glycerol will decrease translation activity.
Phage polymerase (e.g., SP6)	Perform titrations to determine appropriate level.	Use no more than 1µl per reaction because the glycerol present in the enzyme preparation may decrease translation efficiency.

Monitoring synthesis levels is usually accomplished by incorporation of a radioactively labeled amino acid. The amino acid which is chosen for labeling is dependent upon the protein of interest. ^{35}S-methionine is usually chosen due to cost and the stronger emission given by ^{35}S over ^{14}C amino acids. If the number of methionines is low (<5 residues), consider using ^{14}C-leucine. If a functional assay is to be used, use a complete amino acid premix.

Tips for S30 reactions

- If truncated products appear, use a prolonged incubation to determine whether it is the result of degradation or premature termination.
- The premixes minus an amino acid functions to increase the specific activity. It does not mean that the S30 extract completely lacks a specific amino acid.
- If the protein contains a large number of the amino acid used for labeling (e.g., methionine) and the amount of label is used up too quickly, premature termination may result and truncated products will be observed.
- Reactions are usually performed at 30°C, but incubation at 25°C for an extended period (2-6 hours) may increase synthesis levels.
- Cleavage of the coding sequence at a particular site with concomitant disappearance of a protein band is a confirmation of that band's identity.
- Some protein processing of β-lactamase has been observed.

In Vitro Translation of Proteins

VI. References

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VII. Ordering Information and Additional Literature Available from Promega

Ordering Information

Rabbit Reticulocyte Lysate

Product	Size	Cat.#
Rabbit Reticulocyte Lysate	1ml	L4960
	5ml	L4970
	10ml	L4980
Untreated Lysate	1ml	L4151
	5ml	L4152
	10ml	L4153

TnT Coupled Transcription/Translation Systems

Product	Cat.#
TnT™ SP6 Coupled Reticulocyte Lysate System	L4600
TnT™ T7 Coupled Reticulocyte Lysate System	L4610
TnT™ T3 Coupled Reticulocyte Lysate System	L4950
TnT™ SP6 Wheat Germ Extract System	L4130
TnT™ T7 Wheat Germ Extract System	L4140
TnT™ T3 Wheat Germ Extract System	L4120

TnT Rabbit Reticulocyte Lysate and Wheat Germ Extract are supplied on 200µl aliquots. Each 1ml order also includes the specified RNA Polymerase, three Amino Acid Mixtures (Minus Methionine, Minus Leucine and Minus Cysteine), and a Luciferase Control DNA, Luciferase Assay Reagents, and Luciferase Assay Wells, sufficient to perform 30 x 50µl reactions.

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VII. Ordering Information and Additional Literature Available from Promega

(continued)

Flexi™ Rabbit Reticulocyte Lysate System

Product	Cat.#
Flexi™ Rabbit Reticulocyte Lysate System	L4540

Flexi Rabbit Reticulocyte Lysate is supplied in 200µl aliquots. Each 1ml order also includes three Amino Acid Mixtures (Minus Methionine, Minus Leucine, and Minus Cysteine), and Luciferase Control RNA and Luciferase Assay Reagents, sufficient to perform 30 x 50µl reactions.

Wheat Germ Extract

Product	Size	Cat.#
Wheat Germ Extract, Minus Leucine	1ml	L4370
	5ml	L4410
Wheat Germ Extract, Minus Methionine	1ml	L4380
	5ml	L4420
Wheat Germ Extract, Minus Cysteine	1ml	L4390
	5ml	L4430
Wheat Germ Extract, Minus Leucine and Methionine	1ml	L4400
	5ml	L4440

Wheat Germ Extract is provided as 200µl aliquots. Each 1ml order also includes 5µg of BMV Control RNA, Potassium Acetate, and a choice of one amino acid mixture.

Canine Pancreatic Microsomal Membranes

Product	Size	Cat.#
Canine Microsomal Membranes	100EQ	Y4041
	500EQ (5 x 100EQ)	Y4051

Each order of Canine Microsomal Membranes contains the specified amount of membranes along with 1µg of Signal Sequence Control mRNA, and 1µg of Core Glycosylation Control mRNA.

Product	Size	Cat.#
Signal Sequence Control mRNA	5µg	Y4061
Core Glycosylation Control mRNA	1µg	Y4071

Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination Systems

Product	Cat.#
Combination System, Minus Leucine	L4340
Combination System, Minus Methionine	L4330
Combination System, Minus Cysteine	L4360
Combination System, Minus Leucine and Methionine	L4350

Each order of the Combination System includes 0.4ml of Rabbit Reticulocyte Lysate, 0.4ml of Wheat Germ Extract, BMV Control RNA, Potassium Acetate.

In Vitro Translation of Proteins

VII. Ordering Information and Additional Literature Available from Promega

(continued)

E. coli S30 Extract Prokaryotic Translation Systems for Circular or Linear DNA

E. coli S30 Extract for Circular DNA

Product	Size	Cat.#
E. coli S30 Extract, Minus Methionine	450µl	L4500
E. coli S30 Extract, Minus Leucine	450µl	L4510
E. coli S30 Extract, Minus Cysteine	450µl	L4520
E. coli S30 Extract, Minus Leucine and Methionine	450µl	L4530

The S30 Extract is supplied as 150µl aliquots. Each system also includes 600µl Premix (complete), 600µl Premix (minus specified amino acids), Control DNA and Control Assay Reagents.

E. coli S30 Extract for Linear DNA

Product	Size	Cat.#
E. coli S30 Extract for Linear DNA, Minus Methionine	450µl	L4880
E. coli S30 Extract for Linear DNA, Minus Leucine	450µl	L4890
E. coli S30 Extract for Linear DNA, Minus Cysteine	450µl	L4900
E. coli S30 Extract for Linear DNA, Minus Leucine and Methionine	450µl	L4910

The S30 Extract is supplied as 150µl aliquots. Each system also includes 600µl Premix (complete), 600µl Premix (minus specified amino acids), Control DNA and Control Assay Reagents.

Amino Acid Mixtures

Product	Size	Cat.#
Amino Acid Mixture, Minus Leucine	175µl	L9951
Amino Acid Mixture, Minus Methionine	175µl	L9961
Amino Acid Mixture, Minus Cysteine	175µl	L4471
Amino Acid Mixture, Minus Leucine and Methionine	175µl	L4481
Amino Acid Mixture, Complete	175µl	L4461

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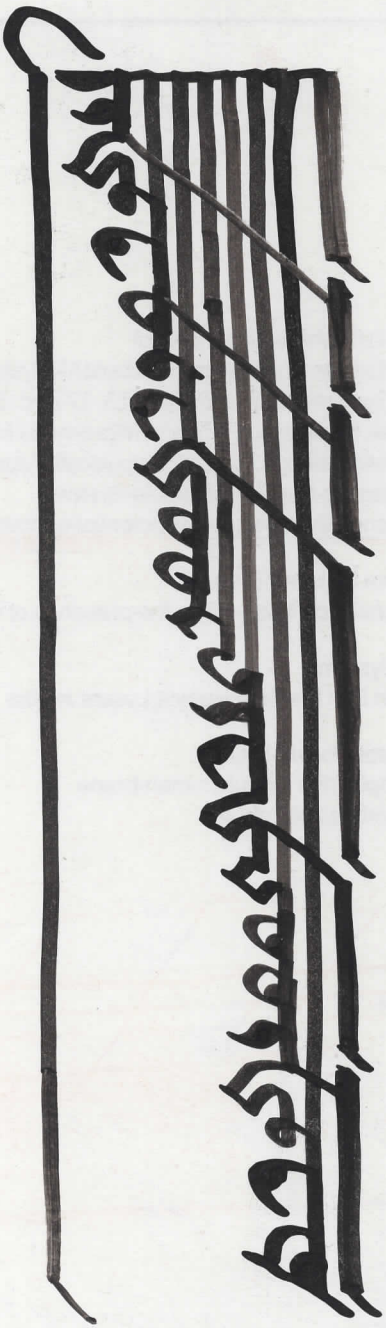
(continued)

Available Promega Technical Literature

Title	Lit.#
Protocols	
E. coli S30 System for linear template	TB102
E. coli S30 System for circular template	TB092
Rabbit Reticulocyte Lysate	TM232
TNT Coupled Reticulocyte Lysate	TB126
TNT Wheat Germ Extract	TB165
Flexi Lysate	TB127
Wheat Germ Extract	TM230
Canine Microsomal Membranes	TM231
***Promega Notes* Articles**	
Coupled *in vitro* transcription/translation using TNT Wheat Germ Extract	PN40
Production of milligram amounts of highly translatable RNA using the RiboMAX System	PN39
TNT Lysate Coupled Transcription/Translation Systems: Comparison of T3, T7 and SP6 Systems	PN38
Post-translational processing: Use of TNT Lysate Systems with Canine Microsomal Membranes	PN38
The TNT Lysate Systems: one step transcription/translation in rabbit reticulocyte lysate	PN35
Optimization of *in vitro* translation reactions using the Salt Select Lysate System	PN35
E. coli S30 update: Sensitivity of coupled transcription/translation to potential contaminants and variation in reaction conditions (circular)	PN32
E. coli Coupled Transcription/Translation System for linear DNA	PN31
Rabbit Reticulocyte Lysate: troubleshooting translation reactions in the presence of chloride and autoradiogram background	PN31
E. coli S30 Coupled Transcription/Translation System	PN26
Post-translational isoprenylation of human lamin B in the Reticulocyte Lysate *in vitro* Translation System	PN26
Cell-free translation of plant mRNA in Rabbit Reticulocyte Lysate	PN19
Assaying protein translocation across the endoplasmic reticulum membrane	PN11
Translation in Wheat Germ Extract of *in vitro* synthesized mRNA	PN9
Data Sheets	
E. coli S30 Transcription Translation	DS044
Wheat Germ Extract	DS005
Canine Microsomal Membranes	DS004
Flexi Lysate	DS105
TNT Coupled Transcription/Translation Systems	DS106

In Vitro Translation of Proteins

Notes



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Purification

Soluble Proteins

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Purification of Protein from *E. coli*

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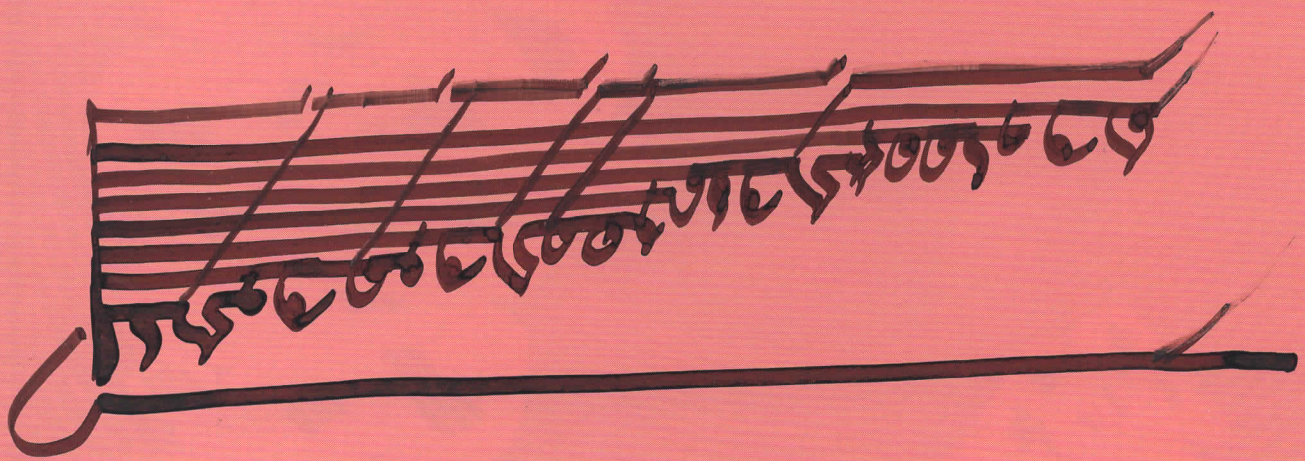

Purification of Protein from *E. coli*

I. Purification of Protein from *E. coli*

Occasionally, a protein may be purified from an overexpression system in a heterologous host, usually *E. coli*. Generally overexpression systems are used because large amounts of protein are required, mutagenesis studies are contemplated (although some of these can be done using *in vitro* translation systems), or the protein copurifies with contaminants when isolated from its natural source. Before designing a purification strategy, decide on the protein amount and purity required for subsequent applications. For instance, if a large amount of protein of moderate purity is required, different techniques will be considered than if a small amount of high purity protein is desired. Table 1 presents some of the requirements for applications which may use purified protein.

In general, the purification scheme is of less interest than obtaining the purified protein itself for experiments, pushing for quick development of a purification protocol. Because each protein has its own unique characteristics, no standard protocol is available. Success will then depend on the rapid determination of the protein's characteristics and behavior under various conditions. An empirical approach to securing the information about solubility, stability, and retention behavior on chromatographic resins often greatly speeds up the development of a protein purification process.

Table 1. Applications Requiring Purified Protein.

Technique	Average Amount Required	Purity/Activity of Isolated Protein
Crystallography	100mg	very high
Enzyme kinetics	10mg	moderate
Antibody production	1mg	low/moderate
Multiprotein enzyme complexes	1-10µg	high
Mutagenesis studies	1-2mg	moderate

Tips about protein purification from E. coli

- Each protein has unique properties, many of which cannot be predicted from the amino acid sequence. Learn these properties as the steps in the purification protocol are developed and use them in subsequent steps. Do not try to force separations to work.
- Plan to culture and lyse a liter of cells daily for 5-10 days.
- Use the first few preps to learn about a particular protein's properties, not to produce active material. When finished with the prep, discard all fractions.
- For each subsequent prep, move the purification protocol rapidly through the steps already developed, then spend time optimizing the next step.

Purification of Protein from *E. coli*

I. Purification of Protein from *E. coli*

(continued)

A flowchart of the processes is presented in Figure 1. This chapter covers optimization of protein expression, cell breakage, and how to proceed if the protein is soluble after cell breakage. Chapter 7 discusses ways to isolate inclusion bodies and refold proteins from them.

The purpose of this chapter and the following chapter is not to define a purification protocol clearly, but to provide the basic guidelines for developing a protocol or deciding to select another system for protein purification.

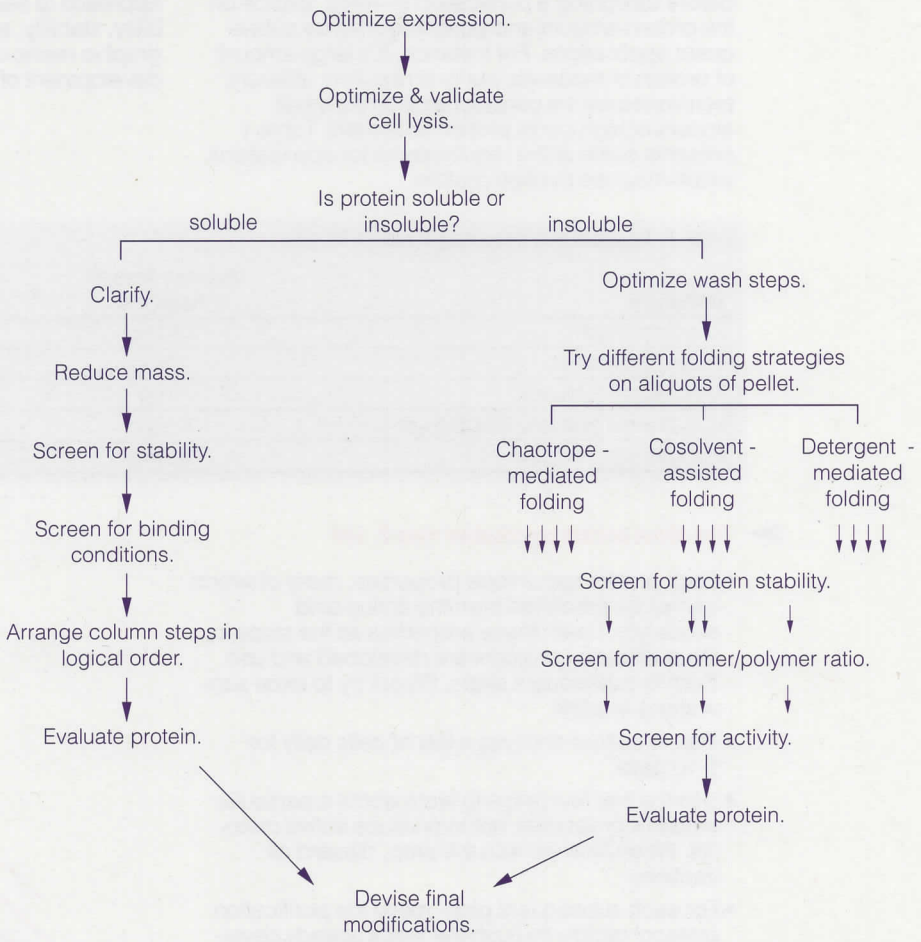

Figure 1. General overview of the development of protein purification protocols.

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II. Materials and Methods Required

Equipment

Many laboratories will have much of the equipment necessary for protein purification. Table 2 presents a checklist of this equipment. Depending upon the special needs of your assay system (see "Monitoring the purification" in this chapter), a variety of special equipment needs, such as scintillation counters or photographic equipment, may be necessary. Before buying expensive equipment, determine if other less costly alternatives are available or if it is possible to borrow equipment from colleagues. Remember, while there may be sophisticated pieces of equipment available, the simplest ones are usually the most effective.

The following equipment is *not* essential, but might prove helpful:

- **Conductivity meter** - Useful in monitoring gradient quality and defining actual salt concentration at which the protein elutes.
- **Ultrafiltration apparatus and membranes** - For the concentration of dilute protein solutions. Can also be used as a method of buffer exchange.

Chemicals

The most important chemical used is water. Use the best grade available, and at a minimum use deionized water. Any attempt to use regular tap water may cause severe problems due to the presence of heavy metal ions and other contaminants.

When choosing a source for chemicals, it is best to purchase high quality reagents from a reliable vendor with a good reputation and a consistent product. Table 3 contains some basic chemicals that may be useful in your protein purification scheme.

Table 2. Equipment for Protein Purification.

Equipment	Comments
pH meter	Use a meter accurate to two decimal places. Do not use pH paper. Accuracy of pH measurements is critical to reproduce purification techniques.
Spectrophotometer	Should measure ultraviolet and visible light. For activity assays, the spectrophotometer may need to be temperature controlled and have kinetic capabilities.
Centrifuge	The centrifuge must be refrigerated and capable of generating >10,000 x g. Centrifuge size is dependent upon the scale of the purification performed.
Assortment of chromatography resins and columns	Sizes and types depend on the scale of work performed.
Miscellaneous	Stir plates and stir bars. Ice and/or cold box. Pipets, test tubes, quartz cuvettes, beakers, graduated cylinders, centrifuge tubes. Dialysis tubing.
Electrophoresis units	For running SDS-PAGE or native polyacrylamide gels. Precast gels are useful for rapid methods development.

Purification of Protein from *E. coli*

II. Materials and Methods Required

(continued)

Table 3. Chemicals and Reagents for Protein Purification*.

Chemicals/Reagents		Comments
Basic Buffer Salts	Tris	Watch for trace metals in buffer salts.
	Potassium phosphate (mono- and dibasic)	
	Sodium phosphate (mono- and dibasic)	
Solutions	Hydrochloric acid	
	Sodium hydroxide solution	
	Phosphoric acid	
	Polyethyleneimine (PEI)	Concentration varies by suppliers.
	β-mercaptoethanol	Store cold and completely sealed.
	Triton® X-100	Trace reductants or oxidants may be present.
	Ethanol	Purchase in glass containers to avoid trace metals.
Other Reagents	Sodium chloride	
	Potassium chloride	
	EDTA	
	Ammonium sulfate	Use with a minimum of 40mM buffers.
	Sodium deoxycholate (DOC)	Viscous when dissolved.
	Protease inhibitors	Expensive, use sparingly.
	Glycerol	Purchase in glass containers to avoid trace metals.
	Dithiothreitol	
	Bovine serum albumin (BSA)	

*Always know the hazards involved with the use of each chemical by referring to its accompanying Material Safety Data Sheet (MSDS). Use proper handling and disposal procedures.

Buffer preparation

Most enzymes are stable and active at neutral pHs (between 7.0 and 7.5) and can usually be purified using either of the following buffer systems:

1. 20-50mM potassium or sodium phosphate, pH 7.0
2. 10-100mM Tris-HCl, pH 7.5

Sometimes, however, neither of these buffer systems will be optimal for either the activity of your protein or for its purification, and another must be chosen.

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II. Materials and Methods Required

(continued)

When selecting a buffer for purification trials, remember three important factors:

pK_a of the Buffer - The pK_a is the pH at which the concentration of the acidic form of the buffer ion is equal to the concentration of the basic form. The buffering capacity of a system is strongest at this pH. Generally, try to work within 0.5 pH units of a buffer's pK_a (in some cases, 1.0 pH unit) (see Appendix).

Ionic Strength - The ionic strength of a buffer will proportionally affect its buffering capacity. For example, 5mM Tris-HCl, pH 7.4 would have approximately 10 times less the buffering capacity and would be much more susceptible to pH change than 50mM Tris-HCl, pH 7.4. As a rule, the further away from the pK_a a buffer is prepared, the stronger the ionic strength must be. Usually use in the range of 10-100mM.

Effect of Temperature - Some buffer systems, such as Tris-HCl, can be greatly affected by a change in temperature while others, such as phosphate buffers, are only slightly affected. Consult a buffer table to determine how temperature affects the system and adjust accordingly (see Appendix).

Other factors to consider are:

- Do the buffer ions interact with other components in the system (e.g., Ca^{2+} is incompatible with PO_4^{2-})?
- How expensive is the buffer?
- How soluble is the buffer?
- Will this buffer affect the assay system (e.g., UV absorbance, activity)?

To avoid loss of protein activity by denaturation, we recommend adding stabilizing compounds to all of the buffer systems. Table 4 provides a list of common stabilizers and their uses.

Table 4. Common Stabilizers for Protein Purification Buffers.

Stabilizer	Concentration	Comments
EDTA	0.1-1.0mM	Will complex with heavy metal ions that may inactivate the protein. Helps to inactivate some proteases that may be present.
β-mercaptoethanol (BME) or dithiothreitol (DTT)	5-20mM (BME) ~1mM (DTT)	Helps to avoid unwanted disulfide bond formation. DTT lasts longer and does not form adducts, but costs more than BME. BME decays within 24 hours, forms adducts and costs less than DTT.
Sodium chloride or potassium chloride	minimum 50mM	Maintains the buffer's ionic strength near the physiological ionic levels. If the salt concentration is too high, proteins may not absorb to chromatographic resins.
Glycerol	5-20%	Not all proteins are stabilized by glycerol, and some are actually destabilized by its presence. Glycerol's greatest effect is on a protein's stability to freeze/thaw.
Protease inhibitors	varies	The choice of protease inhibitor is dependent on the type of proteases present (see Appendix). Often protease inhibitors will only be necessary during the initial steps.

Purification of Protein from *E. coli*

II. Materials and Methods Required

(continued)

Tips for buffer preparation

- Buffers should always be stored refrigerated to avoid microbial growth.
- Use BSA or detergents such as Triton X-100 or Tween 20 for dilute protein solutions to avoid losses due to absorption of the protein to container surfaces.
- Retest the stability of the protein in various buffers (vary pH, salt concentration, stabilizers, etc.) after some purification.
- If the protein of interest requires an essential metal ion, then work with very pure buffer salts that are free of contamination by heavy metals or use a complexing agent with a different selectivity.

Monitoring the purification

Before developing a purification protocol, methods to monitor the results must be in place. First, an assay for total protein is necessary (e.g., Bradford or BCA), and thought should be given to using a 96-well format for ease. Next, a method(s) to track the protein of interest is required and can take a wide variety of forms. If antibodies are available, Western or dot blots can be used (see Chapter 3). In some instances, SDS-PAGE will suffice (see Chapter 2). Finally, if the desired protein has some enzymatic activity, activity or functional tests can be developed. One strength of the *Western Express*™ dot blot protocol is that it can locate the protein of interest faster than most other methods (less than 1 hour). This method can limit the number of samples that must be checked by other methods such as activity assays.

Whatever route is chosen, remember that the conditions of the isolation step may affect the subsequent assay. If the purification solutions affect the accuracy of the assay (or are suspect), dialyze part of the sample prior to the assay.

III. Optimizing Expression

Prior to purification, optimize expression levels of the desired protein to a minimum of 2-3% of the total protein. At this expression level the protein should clearly appear as a new band on SDS-PAGE after induction, but the band would be no more prominent than some heavier bands already present. Even if this level is obtained, it is worthwhile to vary conditions to boost expression levels more. Sometimes changing a strain is all that is needed to boost expression. Table 5 provides various growth parameters that may affect the level of expression.

Tips on optimizing growth conditions

- In general, store all strains frozen as glycerol stocks. Prepare these stocks using cultures at an A_{660} of approximately 0.1.
- Seed cultures for induction and analyze conditions (e.g., strains, additives) in multiples of two.
- For the T7 system developed by Studier, a currently popular expression system, try 0.5-1.0mM IPTG and IPTG with rifampicin (150µg/ml, added 30 minutes after IPTG) for induction.

Table 5. Growth Parameters Affecting Protein Expression in *E. coli*.

Parameter	Comments
Strain	Varying strains can increase expression levels and trying numerous strains is recommended. For example, with the T7 expression system, BL21DE3 (*plys*S) is better for the production of toxic proteins than BL21DE3 (1).
Media	Always use a rich medium, such as L broth.
Induction Time	Induce at an A_{660} of 0.4-0.6. (mid-log phase); monitor 1-4 hours post-induction.
Temperature	Altering growth temperatures may affect the accumulation of protein.
Promoter	Know the inducers and repressors of the chosen expression system. Different promoters can have different strengths.

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III. Optimizing Expression

(continued)

In a typical experiment, a fresh overnight culture is used to seed a 50ml culture for induction. Before induction, remove a 1ml sample and collect cells by centrifugation. After induction, remove and collect cells from 1ml samples periodically for 1-4 hours to monitor the accumulation of the desired protein.

Resuspend cells immediately after collection in 4X SDS loading buffer (approximately 100µl per A_{660} unit), heat at 95°C for 5 minutes, and analyze 5µl on a miniformat SDS-PAGE gel (see Chapter 2). After 4 hours, harvest cells and lyse to determine if the desired protein is insoluble.

IV. Purification of Soluble Proteins

Cell lysis

The first step in developing a purification protocol will be to determine an efficient method to lyse the *E. coli* strain to release the protein. As a minimum, start with approximately 1-2 liters of culture resuspended in a minimum of two volumes of the extraction buffer (i.e., 100 grams of cells in 200ml of buffer). Table 6 presents a number of cell lysis alternatives.

Table 6. Methods to Lyse Cells.

Type	Comment
Enzymatic	Example: lysozyme. Can be expensive. Possible source of contamination.
Chemical and enzymatic	Examples: Triton X-100, sodium deoxycholate (DOC). Must empirically determine appropriate starting concentration for a cell line. Use in combination with lysozyme.
Mechanical	Examples: Sonication, French Press, bead grinding. Homogenization of the sample can be a problem when using a French Press.

A number of methods exist to determine the degree of cell lysis and a few of these are described in Table 7.

Table 7. Methods to Monitor Lysis.

Method	Procedure
Reduction of whole cells	Compare the A_{660} before and after treatment. Lower A_{660} indicates successful lysis.
Monitor nucleic acid release	Compare the A_{260} before and after treatment. Increase in the A_{260} indicates an increase in the nucleic acid concentration, demonstrating successful lysis. This method could be difficult because of the "haze" generated, which can alter absorbance readings.
Microscopically	Compare cells before and after treatment.

Purification of Protein from *E. coli*

IV. Purification of Soluble Proteins

(continued)

Tips for cell lysis

- Keep the sample cold during the lysis process. If the sample heats up, proteins may be inactivated.
- Resuspend solid mass (cell pellet) in a minimum of two volumes of extraction buffer.
- Check the pH of the sample after lysis. The pH may alter due to the induction method and may affect protein stability.
- A common extraction buffer consists of Tris-HCl, pH 7.5 (or potassium phosphate, pH 7.0), 1mM EDTA, 100mM NaCl, 5% glycerol, 0.1% Triton X-100 (or 0.2% DOC), 1mM DTT.

Clarification: Nucleic acid removal

The first step after cell lysis is to remove nucleic acid and cellular debris from the preparation. This can be achieved by the addition of polyethyleneimine (PEI), streptomycin sulfate or polyethylene glycol, all of which precipitate nucleic acids from solution (2). The concentration for a particular system will have to be determined empirically. PEI and streptomycin sulfate precipitate by titration; PEG by concentration. The subsequent precipitate is then pelleted by centrifugation in a preparative centrifuge (approximately 10,000 x g for 30-40min).

Tips for clarification

- The use of PEI requires a minimum of 250mM Na^+ in the buffer to avoid precipitation of proteins.
- PEG is less desirable because it can also precipitate some proteins.
- Some precipitants may interfere with subsequent assays for the protein. If necessary, dialyze the sample to remove the precipitant, or try a different precipitant.

Mass reduction/protein precipitation

This step removes the unwanted proteins and other contaminants present in the preparation. Numerous approaches exist, and some of these are discussed in Table 8. As with all purification steps, the correct method and its parameters will vary with the protein isolated.

After the precipitation, test the supernatant for the presence of the desired protein. This may require dialysis of the sample to normal conditions because the precipitation conditions may affect the assay.

Tips for mass reduction

- Always add PEG slowly and with stirring to avoid localization of salt/solvent/PEG.
- Always keep the solution cold during the precipitation.

Table 8. Methods to Precipitate Proteins.

Ammonium sulfate	Most common method. Titrate at 4°C by adding ammonium sulfate to produce fractions of 0-40%, 40-60% and 60-80% (see Appendix). Watch for buffering; need at least 40mM.
Heat denaturation	Heat between 30-70°C.
PEG/salt precipitation	Use fresh PEG. The appropriate MW, as well as the appropriate salt concentration, will have to be determined empirically.

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IV. Purification of Soluble Proteins

(continued)

Table 9. Common Chromatography Resins.

	Cation exchangers	Anion exchangers	Hydrophobic interactions
weak	CM = carboxymethyl	DEAE = diethylaminoethyl	butyl
strong	SP = sulfopropyl	Q = quanternary anion	octyl
			phenyl*

* also may have aromatic selectivity.

Chromatography

The next step is to determine appropriate chromatography conditions for the protein. One common mistake here is to immediately turn to expensive affinity resins. Instead, test a variety of resins to find the best (see Table 9). The goal is to identify the appropriate resin and salt conditions to meet one of the following criteria:

a. The protein does not bind to the resin at a particular salt concentration, but binds the majority of undesired protein in the mixture.

b. The desired protein binds the resin at a particular salt concentration when a large amount of undesired protein does not bind.

Test tube tests for chromatographic conditions

To empirically determine chromatography resins and conditions to use, perform "test tube" tests rather than running small columns. (All steps and solutions should be at 4°C.)

1. Wash 5ml of the test resin several times using 50ml of a low salt buffer (20-50mM buffer pH 7-8) in a screw cap tube. Resuspend the resin in 5ml of low salt buffer. Dispense 200µl aliquots into five to ten 1.5ml microcentrifuge tubes. Let the resin settle and then remove excess buffer.
2. To each tube, add 500µl of cell lysate.
3. Prepare high salt buffer by adding NaCl to the low salt buffer so the final salt concentration is 2M.
4. Add low and high salt buffer in combination to each tube to a final volume of 1ml (combined total of 400µl) and vary this combination such that the NaCl concentration ranges from that of the starting buffer (20-50mM) to 0.5M NaCl.
5. Invert the tubes 5-10 times to mix. Allow resin to settle. Repeat this step two more times over a 15-minute period.
6. Centrifuge briefly. Determine the amount of total protein (compared to the total applied) and the desired protein in the supernatant. Plot this data to generate a graph such as shown in Figure 2.

Purification of Protein from *E. coli*

IV. Purification of Soluble Proteins

(continued)

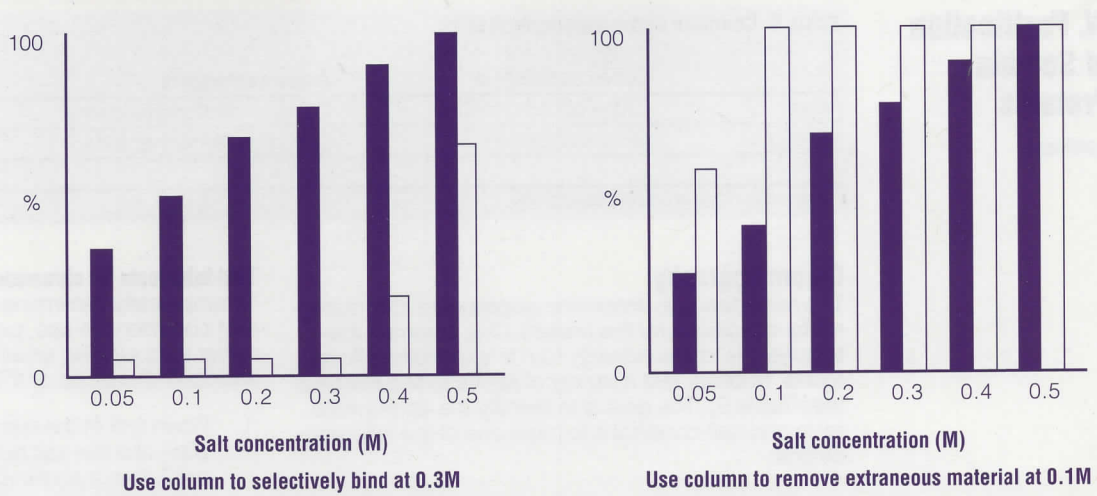

Figure 2. Sample chromatography results.

Tips for chromatography

- If the protein does not elute from a hydrophobic resin at 0M NaCl, then repeat the test using 20% glycerol in all solutions.
- When determining the order of chromatographic steps, take advantage of the properties required to elute the protein from the various resins. For example, apply the collected samples from an ion exchange column eluted in high salt onto a hydrophobic interaction resin which requires high salt for binding. Then apply the collected fractions from the hydrophobic resin (eluted in low salt) to another ion exchange resin, requiring low salt for binding.
- Consider ammonium sulfate precipitation of fractions collected from an ion exchange resin, followed by dialysis and then application to a size exclusion resin (e.g., Sephadex® or Sepharose® G-150) which will finish desalting the material. Do not, however, use a size exclusion resin until the material has been through two chromatographic purifications.
- Try the least expensive resins first.
- Do not assume a resin will not work until you try it.
- Remember that binding to a resin is buffer/pH dependent.

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IV. Purification of Soluble Proteins

(continued)

- All purification steps should be carried out at refrigerated temperatures (approximately 4-8°C) or on ice. Avoid freezing protein solutions. Many enzymes lose activity when put through a freeze/thaw cycle.
- As a rule, a concentrated protein solution will show better stability than a dilute one. If possible it may be helpful to concentrate a dilute solution to improve its stability. Another method of stabilizing very dilute protein solutions is by the addition of bovine serum albumin (BSA). Great care should be taken, however, to ensure that the BSA does not contain anything that may contaminate the protein of interest.
- Resins that do not bind protein could alternatively be used to remove contaminants from the prep.

Potential Problems with Proteins Purified from *E. coli*

For information, please see Table 10 in Chapter 7.

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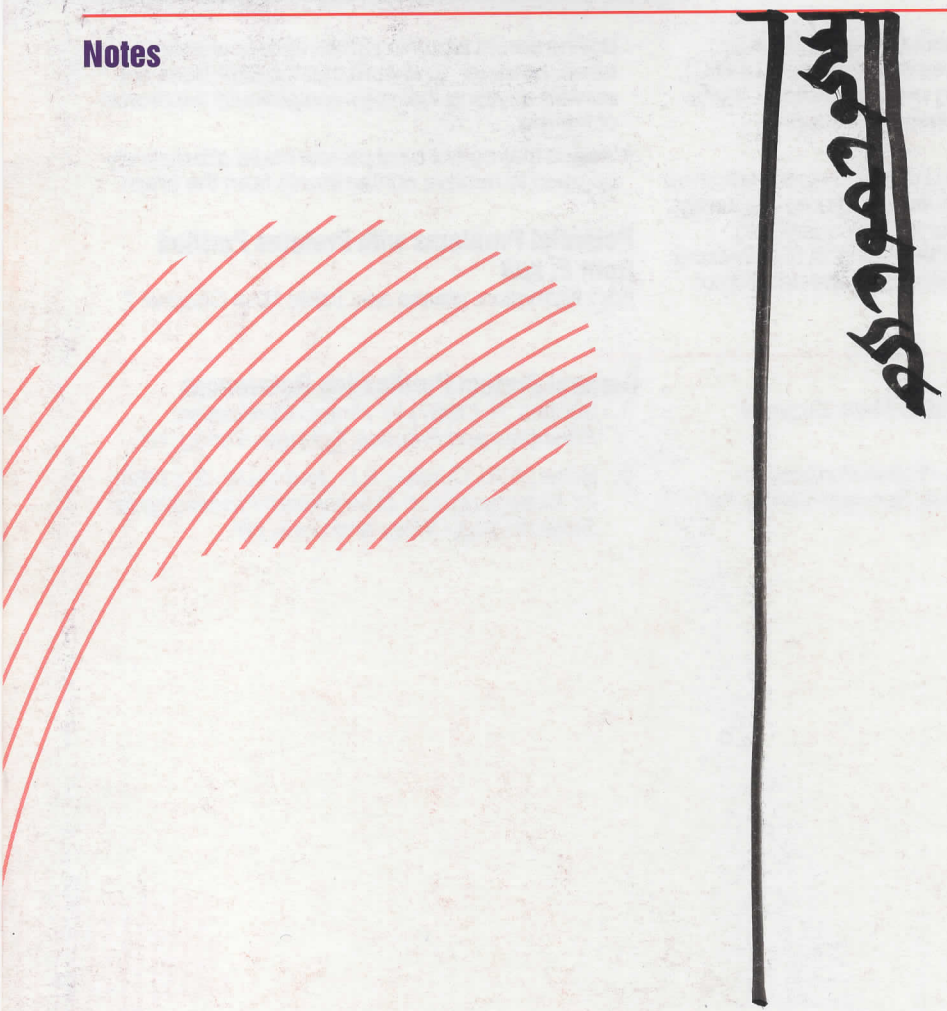
Purification of Protein from *E. coli*

Notes



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7 Purification

Inclusion Bodies & Protein Refolding

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Purification of Inclusion Bodies and Refolding

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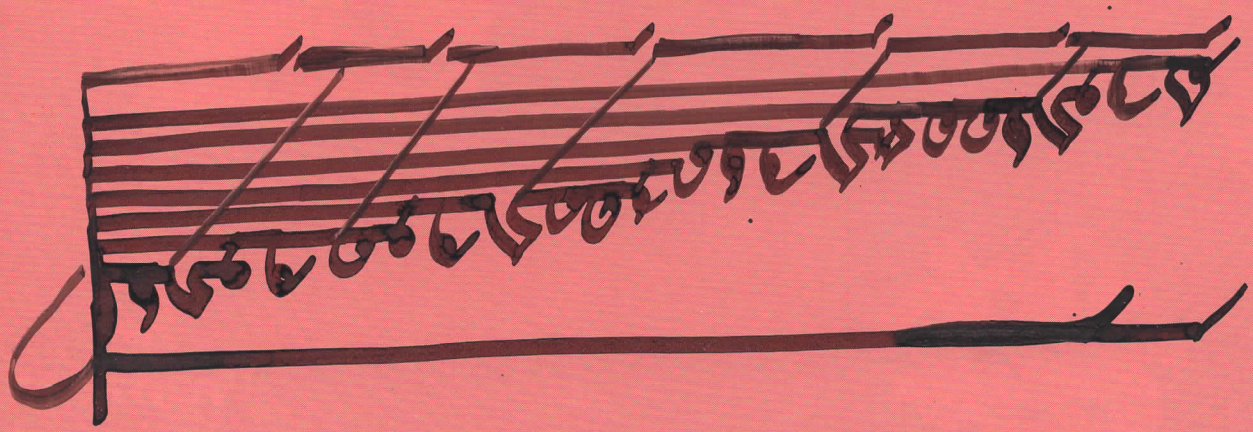

Purification of Inclusion Bodies and Refolding

I. Purification of Inclusion Bodies and Refolding

At times expression of a gene in *E. coli* will produce an insoluble protein which forms inclusion bodies. Inclusion bodies provide a means to easily and rapidly isolate protein. If, however, active protein is required, the protein in the inclusion body must be refolded.

It is possible to set up a series of experiments over a two-week period that can indicate whether the protein can be successfully refolded, or whether it is best to express the protein in an alternative production system (e.g., baculovirus, CHO cells). Refolding protocols are best determined empirically, as with fractionating a soluble protein described in Chapter 6. Because of the complexities involved in refolding proteins from inclusion bodies, we can only present some basic guidelines. More detailed information may be obtained from courses such as that offered at Cold Spring Harbor Laboratories.

There are actually several (usually overlooked) advantages to having proteins produced in inclusion bodies:

- Simplifies purification (wash isolated inclusion bodies)
- Reduces proteolysis as long as *Omp*T$^-$ strains are used
- Large quantities of starting material can be made
- Proteins can be rapidly isolated
- Simplifies pyrogen removal

The purification and recovery of active protein from inclusion bodies involves several basic steps (Figure 1). The critical points in each step will be addressed in this chapter.

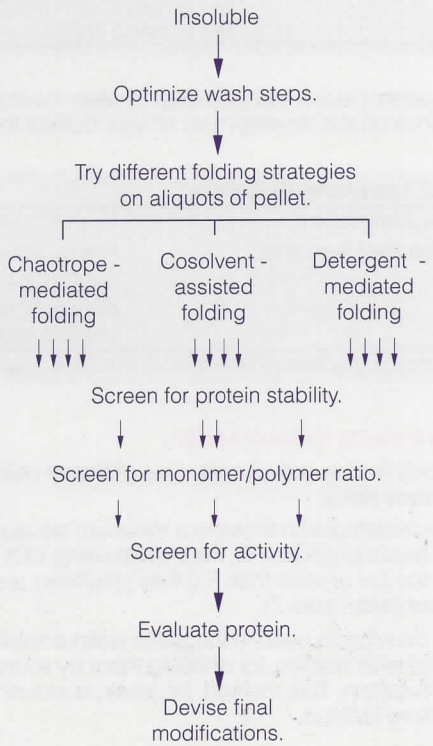

Figure 1. General overview of the development of protein purification protocols.

Purification of Inclusion Bodies and Refolding

I. Purification of Inclusion Bodies and Refolding

(continued)

Cell lysis and clarification

Once growth and expression conditions have been optimized (see "Growth of Cultures," Chapter 6), the isolation of inclusion bodies is relatively simple, requiring only cell lysis, removal or digestion of nucleic acids, and a wash of the protein pellets. Table 1 presents two general isolation schemes.

Table 1. Methods for Preparing Inclusion Bodies.

Step	Method 1	Method 2
Cell lysis	Sonication on ice in buffer containing a detergent. Manton Gaulin homogenizer for large-scale purifications.	Lysozyme treatment followed by treatment in 0.2% sodium deoxycholate (DOC) or 1% Triton X-100. If using *plys* S or *plys* E strain, lysozyme is not required.
Clarification	Shearing (completed during sonication) or Manton Gaulin for large-scale purifications.	DNase/RNase treatment.
Wash	First in 10-20 cell volumes of 2% DOC (pH >8.0) or 1% Triton X-100, then in refolding buffer minus solubilization agent.	First in 10-20 cell volumes of 2% DOC (pH >8.0) or 1% Triton X-100, then in refolding buffer minus solubilization agent.

It is important in the cell lysis step to keep the buffer simple and to work quickly. Table 2 provides some guidelines on the development of lysis buffers for inclusion body isolation.

Table 2. Lysis Buffer Guidelines*.

General formulation:	Tris-HCl, NaCl, EDTA, plus protease inhibitors if desired.
Disulfide bond formation:	Not required – add DTT to 1-2mM.
	Required – add no reductant and maintain the pH at less than 7.5 after deoxycholate wash step. Also include high concentration of EDTA to chelate metals, especially copper.

* For more information on development of buffers, see the Buffer Preparation section in Chapter 6.

Tips for isolating inclusion bodies

- Use only freshly grown cells. Use of frozen cells will decrease yields.
- Keep centrifugation times to a minimum because DOC tends to gel at 4°C. Also when using DOC, keep the pH greater than 8.0 if no disulfides are present (see Table 2).
- If the detergents used in the pellet wash solubilize the inclusion bodies, try isolating them by sucrose centrifugation. This method, however, is slower and more tedious.

At this point, the protein is 75-95% pure. To obtain protein from the inclusion bodies, they must be solubilized using reagents such as guanidine salts, urea, or detergents. This treatment results in a denatured protein which may be suitable for some applications. If active protein is required, however, the next step in this purification strategy is to determine the correct refolding conditions empirically.

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II. Refolding

Protein folding intermediates

A basic theoretical understanding of the refolding process is helpful before discussing refolding strategies. In general, hydrophobic forces maintain the protein in the inclusion body. The agents used to solubilize inclusion body protein alter or neutralize these forces. The two agents most often used for solubilization are 1) chaotropic agents, such as urea and guanidine salts; or 2) detergents. Chaotropic agents work by changing the ordered structure of water around the protein's hydrophobic regions, making it less favorable for these regions to associate. With detergents, the hydrophobic group of the detergent interacts with the hydrophobic regions of the protein. These interactions result in the detergent's polar or charged "head group" interacting with the bulk water.

As these denaturants are gradually removed, the hydrophobic forces will reappear, pushing the protein through several intermediate states before achieving the native monomeric structure. The formation of these monomeric states is in equilibrium with the formation of undesirable multimers and aggregates. Multimers are complexes consisting of greater than 8 monomers, which are soluble, but often inactive. Aggregated forms are very large complexes of peptides and are insoluble. The key to successful refolding is to create an environment in which most of the protein moves through the intermediate stages as a monomer.

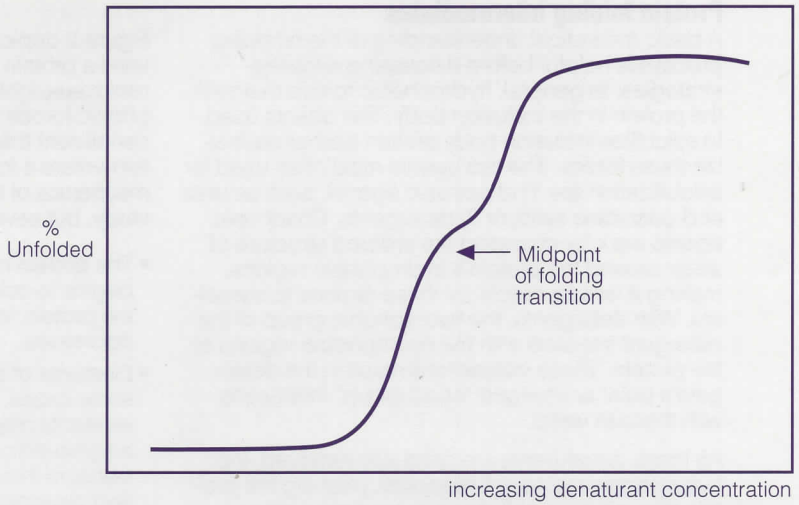
Figure 2 depicts a simple model of the folding transitions a protein undergoes. As denaturant is slowly removed (right-hand side of the graph), the hydrophobic forces strengthen. At some concentration of denaturant that is unique for each protein, the protein enters a folding transition zone. The actual mechanics of this transition are a matter still under study, but several things are clear:

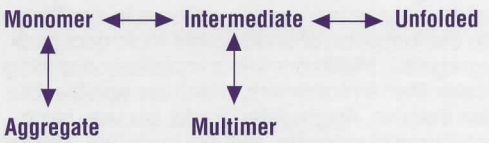
- The protein moves from a random coil state and begins to collapse on itself. The apparent size of the protein monomer (hydrodynamic radius) decreases.
- Elements of secondary structure start to form. In some cases, these nascent secondary structural elements may form hydrophobic faces (e.g., amphipathic helices). These structures are not stable in this portion of the folding curve, but form and disappear rapidly.
- Other hydrophobic secondary structural elements with which the early forming secondary structural elements normally pair, transiently appear, and interact in a "native-like" way. These paired secondary structures are likely to stabilize and persist.
- If the correct hydrophobic association partners do not appear, the nascent secondary structures are likely to interact with the other hydrophobic surfaces to form incorrect intra- or intermolecular associations. Over time these associations are usually less stable than, and will be outcompeted by the correct patterns.
- The stabilization of aggregated intramolecular network associations can result because of the large numbers of monomers that can participate in these associations. Thus even though the individual incorrect associations in a multimer or aggregate are probably weaker than the "correct" ones, in aggregate they can compete with the monomeric folding pathway.

Purification of Inclusion Bodies and Refolding

II. Refolding

(continued)





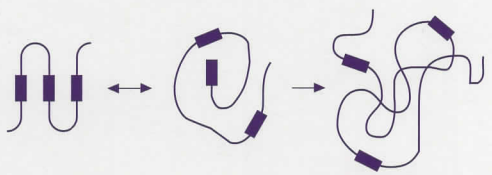


Figure 2. Protein folding equilibrium.

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II. Refolding

(continued)

As a protein goes through the conformational transition from being completely denatured to a properly folded monomer, it passes through what is theoretically described as a transition (or folding) midpoint. It is at this point where multimer/aggregate formation is most marked (1). Table 3 provides a summary of the monomer/multimer equilibrium.

Methods of refolding

Three emerging approaches to refolding proteins are:

- **Chaotrope-mediated folding** - In the presence of chaotropes, the protein is in a semi-rodlike, denatured state. Slow, stepwise removal of the chaotropes disfavors the thermodynamic forces that might otherwise cause hydrophobic portions of the molecule to interact incorrectly.
- **Cosolvent-assisted chaotropic folding** - This method uses regular chaotropic folding protocols but adds PEG, detergents, or other agents to reversibly mask hydrophobic residues that might nucleate multimer formation near the folding midpoint. Cosolvents can be thought to act in the same way as chaperonins.
- **Detergent-mediated folding** - Detergents interact with hydrophobic regions (acting much like chaperonins) and prevent these regions from interacting inappropriately with hydrophobic regions in other protein molecules. This action blocks the conversion of intermediates to multimers. Slow removal of the detergent allows the protein to find the proper conformation.

Table 3. Critical Steps in the Monomer Multimer Equilibrium.

1. Often if multimer/monomer aggregate formation can be blocked, the monomers will slowly find their way to an active state.
2. The multimer/aggregate competition is often most marked near the midpoint of the folding transition.
3. At midpoint of the folding transition, the protein has collapsed into a "molten globule," and some elements of amphipatic secondary structure may have formed.
4. These amphipatic regions must be encouraged to bury in the monomer and not to nucleate multimers.

Purification of Inclusion Bodies and Refolding

II. Refolding

(continued)

Potential advantages and disadvantages of these methods are summarized in Table 4.

Table 4. Approaches to Refolding Proteins.*

Method	Advantages	Disadvantages
Chaotrope-mediated folding	Best documented and familiar. Theoretical underpinnings. Easy to perform.	Requires low protein concentrations (usually <100µg/ml). Expensive, corrosive agents. Must control removal. May have to determine folding midpoint. Expensive for large-scale purifications.
Cosolvent-assisted chaotropic folding	Moderate protein concentration. High success rate to date. Easily adapted from chaotropic folding protocols. Easy to perform. May work at higher protein concentration.	Expensive, corrosive agents. Emerging technique (not many cases reported yet). Must control removal. Expensive for large-scale purifications. Some detergents may associate too tightly to a protein's hydrophobic regions. May have to determine folding midpoint.
Detergent-mediated folding	High protein concentration allowed. Inexpensive, noncorrosive agents. Automatically sets up gradient of removal. High success rate to date. Less expensive. Easy to perform.	Sometimes requires more time than other methods. Less well reported and understood in literature. Need to assay for remaining trace detergent. C-12 SDS is expensive; CTAB and Sarcosyl are not.

*The suggestions we present are for research purposes only. Many of the protocols or variations are covered by patents.

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II. Refolding

(continued)

Whatever protocol is attempted, there are several tips that may help monomer yield irrespective of the protocol used. They are:

- Drop the protein concentration (initially start at <50µg/ml).
- Vary pH.
- Vary temperature (4-25°C).
- For purification of enzymes, try the addition of substrate and/or co-factors.
- Add trace ions.

Screens for success of refolding trials

The amounts of aggregates, multimers, and active and inactive monomers obtained will vary with conditions used. To determine the optimum folding conditions, perform a number of folding trials simultaneously and respectively screen first for solubility, for monomer content, and by activity.

1. **Solubility/Turbidity:** Centrifuge for 5 minutes at >13,000rpm and determine the protein concentration of the supernatant. A high concentration of protein in the supernatant is desirable. Alternatively, measure light scattering of the supernatant by A_{450}. A low absorbance is desirable.
2. **Monomer/multimer ratio:** Analysis of the sample by gel filtration or native gel electrophoresis provides better information about the ratio of multimer to monomer. For gel filtration, choose a resin that does not exclude the monomer, but will exclude polymers. For instance, Sephadex G-100 works well for a protein with MW of 20kDa. Expect the ratio of monomer/polymer to vary with the conditions. Use those conditions which favor monomer formation and test these samples for activity.
3. **Activity:** Perform activity assays after obtaining information from the above tests. For example, an aggregated protein probably is not desired even if it shows activity.

Chaotrope-mediated folding

As described earlier, the multimer/monomer competition is greatest near the midpoint of the folding transition. It is important that at the midpoint, the chaotrope concentration decrease slowly to provide time for proper monomer formation. Methods for determining the midpoint are described in Table 5.

Table 5. Methods for Determining the Transition Midpoint of a Folding Protein.

Method	Technique	Comments
Spectroscopic studies	UV difference. Fluorescence. Circular dichroism (CD).	Useful if equipment available.
Urea gradient gels	Protein is run in a native gel with urea gradient running left to right. The higher the urea concentration, the more the bands spread out. At low urea concentrations, the protein migrates faster than at higher urea concentrations. A smooth change in the apparent size of the protein will be seen, centered on the folding midpoint (1).	Gels difficult to pour in a reproducible manner. Addition of a dye to the urea solution allows one to scan the gel after pouring and determine the actual shape of the urea gradient in the gel.
Turbidimetry	Note shift in absorbance at 600 or 400nm.	Simple to use.

Purification of Inclusion Bodies and Refolding

II. Refolding

(continued)

If the folding midpoint is unknown, proceed slowly through the entire gradient of chaotrope removal. Table 6 presents a sample protocol. Dilution methods are preferred because of their ease and precision. With these methods, however, the protein concentration will also decrease and concentration of the sample may be required later.

Tips for preventing aggregate/multimer formation

- Use only freshly isolated inclusion bodies for refolding experiments.
- Urea can generate cyanate ions which can damage proteins. Use scavengers such as 10mM ethylenediamine to alleviate this problem.
- Work slowly. The major cause of failure in refolding experiments is adding denaturants and then attempting to remove them quickly. This may not provide sufficient time for hydrophobic regions within a protein molecule to associate properly, forcing the equilibrium towards aggregate or multimer formation.
- Perform refolding experiments at low protein concentration (approximately 50-1000µg/ml) to favor monomer formation.

Table 6. Sample Procedure for Chaotropic-Mediated Folding Experiments.*

1.	Prepare washed inclusion bodies and then solubilize in 6M guanidine HCl or 8M urea.
2.	Determine starting protein concentration and then prepare protein stocks at 3 concentrations (0.1, 0.3, 1mg/ml).
3.	Place aliquots of protein in small cylinders and stir continuously.
4.	Add buffer until concentration of chaotrope is approximately 1.5M over the transition midpoint.
5.	Add buffer to decrease the chaotrope concentration in 0.25M increments. Wait 5-15 minutes after each addition.
6.	Repeat step 5 until the concentration of chaotrope is about 1.5M under the transition midpoint.
7.	Dialyze samples overnight.
8.	Screen for solubility and monomer activity.
9.	Refine method and repeat.

* This sample provides a general starting point for refolding and may require optimization for a particular protein.

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II. Refolding

(continued)

If the protocol is successful, a distribution of multimer and active monomer will be obtained with the distribution skewed towards monomer. Results might appear as in Figure 3.

A lower protein concentration for refolding results in more monomeric protein being eluted from the column as determined by protein assay. To improve this ratio, try repeating the protocol, following the steps as suggested in the "Methods of refolding" section in this chapter. Usually, the optimal folding conditions for a preparation are determined by balancing monomer yield against process volumes.

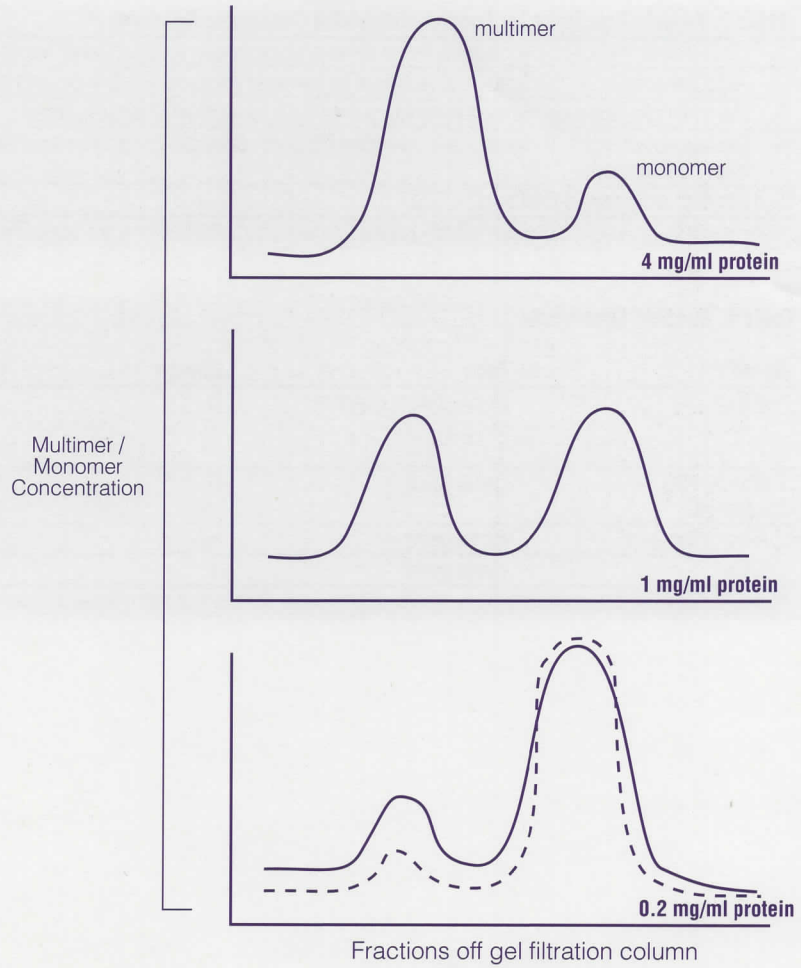

Figure 3. Protein concentration effects on the monomer/multimer ratio. (Hypothetical protein) Solid line is protein concentration, dashed line is associated protein activity.

Purification of Inclusion Bodies and Refolding

II. Refolding

(continued)

Cosolvent-assisted chaotropic refolding

Cosolvent-assisted chaotropic methods use standard chaotropic protocols and solutions, but also add agents to reversibly mask hydrophobic surfaces that form around the midpoint of the folding transition in an attempt to block multimer formation. These "masking" interactions should be strong enough to suppress multimer formation, but not compete with the internalization of hydrophobic patches within the monomer. Examples of such agents are detergents and PEG of defined molecular weight. Detergents seem to work best near their critical micelle concentration (CMC). Table 7 presents a sample protocol.

Table 7. Sample Procedure for Cosolvent-Assisted Chaotropic Refolding.*

1.	Determine the best procedure from simple chaotropic folding (see Table 6).
2.	Add detergent or PEG at desired level to several aliquots (Table 8) and stir continuously.
3.	Perform dilutions as determined from previous chaotropic folding trials.
4.	Dialyze overnight. If necessary, remove PEG or detergent with ion exchange or hydrophobic interaction resin.
5.	Screen and refine method.

* This sample provides a general starting point for refolding and may require optimization for a particular protein.

Table 8. Suitable Cosolvents.

Agent	Type	Charge	Levels to Use
PEG	uncharged polymer	0	Molar ratios of 2-20 to protein; level varies with hydrophobicity of protein.
Triton X-100 Tween 20	detergent	0	CMC*= 600µM. CMC* = 60µM.
Zwittergent 3-16	detergent	+/–	CMC* = ~ 3mM.
CTAB	detergent	+	CMC* = ~ 1mM.

*cmc=critical micelle concentration. Varies slightly as a function of salt concentration.

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II. Refolding

(continued)

Detergent-mediated folding

Detergents such as C-12 SDS, CTAB, or Sarkosyl are used to dissolve and denature inclusion body proteins. When used near their CMC, these detergents effectively coat the transient hydrophobic regions of the protein. Subsequent dialysis sets up a gradual removal of the denaturing agent. At high protein concentrations, the weight ratio of detergent to protein should be approximately 1:1. Table 9 provides a sample protocol for detergent-mediated refolding.

➤ *Tip for refolding methods*

- In some cases only one approach will work, while for others all three methods may provide a satisfactory means to refolding. In other cases nothing may work, and other means for purification should be attempted.

Alternative methods/strategies for refolding

1. **Folding of Immobilized Protein.** In this method, the basis of refolding is to sequester individual monomers. Samples are dissolved in urea and bound to an ion exchange resin (or a chelating resin if protein has a histidine tag). The urea concentration of the column is then decreased, followed by elution of the protein with salt (or histidine).
2. **Addition of Chaperonins.** This method is analogous to cosolvent-assisted chaotropic refolding except various chaperonin proteins (GroEL, GroES) or redox enzymes (thioredoxin, PDI) are substituted for detergents. This procedure is successful sometimes, but the rules are not well-defined. It is also expensive.

Disulfide bonds

Another potential problem to refolding is proper formation of disulfide bonds. Certain precautions should be taken at the cell lysis stage (see Table 2). During the refolding process, the following should be kept in mind:

- Keep the pH low (<7.5) and metals chelated until ready for oxidation.
- To oxidize the protein solution, raise the pH (usually >8), remove EDTA, and add air.
- Additives which may encourage disulfide formation include:
  - metal ions (e.g., copper in low nanomolar range)
  - chemical oxidants
  - disulfide interchange enzymes (PDI, thioredoxin)
- Monitor redox potential with Ellman's Reagent.
- Monitor disulfide formation by reverse phase liquid chromatography (reduced protein elutes last).
- Oxidize the sample until the concentration of free thiols is low (<0.1/protein) then concentrate the protein.

Table 9. Sample Procedure for Detergent-Mediated Refolding.*

1.	Prepare washed inclusion bodies and solubilize in 0.2-0.6% Sarkosyl or CTAB or 0.2% C-12 SDS.
2.	Determine starting protein concentration and prepare protein stocks at 3 concentrations (0.1, 0.3 and 1mg/ml).
3.	Place proteins in dialysis tubing with a molecular weight cut off high enough to retain the protein.
4.	Dialyze overnight or longer against buffer lacking detergent.
5.	Assay for detergent removal.
6.	Screen and refine method.

* This sample provides a general starting point for refolding and may require optimization for a particular protein.

Purification of Inclusion Bodies and Refolding

III. Potential Problems with Proteins Isolated from *E. coli*

All isolated protein should be characterized irrespective of whether it was isolated from the soluble or insoluble fraction. Most common quality problems with proteins folded from inclusion bodies are also present in proteins purified from a soluble fraction.

Table 10 lists some of these problems. Note that all those listed affect both inclusion body protein and soluble protein except carbamylation, which affects only inclusion body protein.

Table 10. Potential Problems with Proteins Purified from *E. coli*.

Potential Problem	Detected by	Comments
Mixed intermolecular disulfides	Tryptic or other mapping	
Deamidation	Tryptic or other mapping, IEF*	Increases with storage at pH>7.5.
Norleucine incorporation for methionine	Tryptic or other mapping, amino acid analysis	Most common with high level expression.
Translation errors	Hard to detect	
Lack of post-translational modification		Assume no phosphorylation, glycosylation.
Partial proteolysis	Tryptic or other mapping, IEF	Less in inclusion bodies.
N-terminal methionine	Tryptic or other mapping, IEF	Some *E. coli* strains remove the methionine.
Carbamylation (urea folding)	IEF	Include scavengers (e.g., ethylenediamine).
Oxidation of other amino acids	Tryptic or other mapping, IEF	More prevalent in refolded samples.
Needs cofactor or subunit		
Multimers	Gel filtration; decreased yield on subsequent chromatography	Very common reason for low specific activity.

* IEF = Isoelectric focusing

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IV. References

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2. Creighton, T.E. (1986) *Meth. Enzymol.* **131**, 156.

Very little data exists which directly addresses the preparative folding problem. For this reason, we provide you with references from three authors (David Brems, Daniel Wang, and Paul Horowitz) whose work demonstrates many of the principles stated herein.

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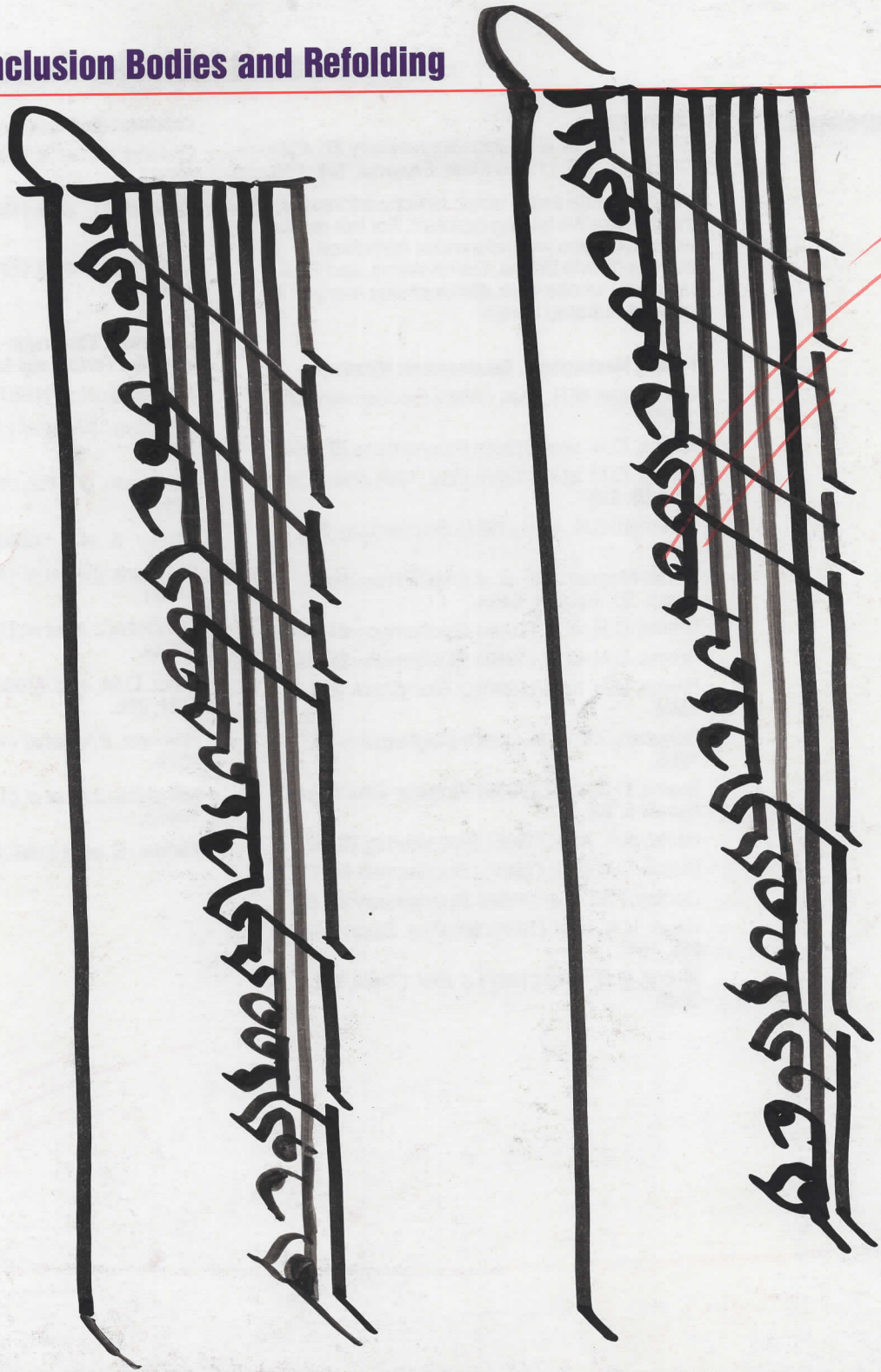
Purification of Inclusion Bodies and Refolding

Notes



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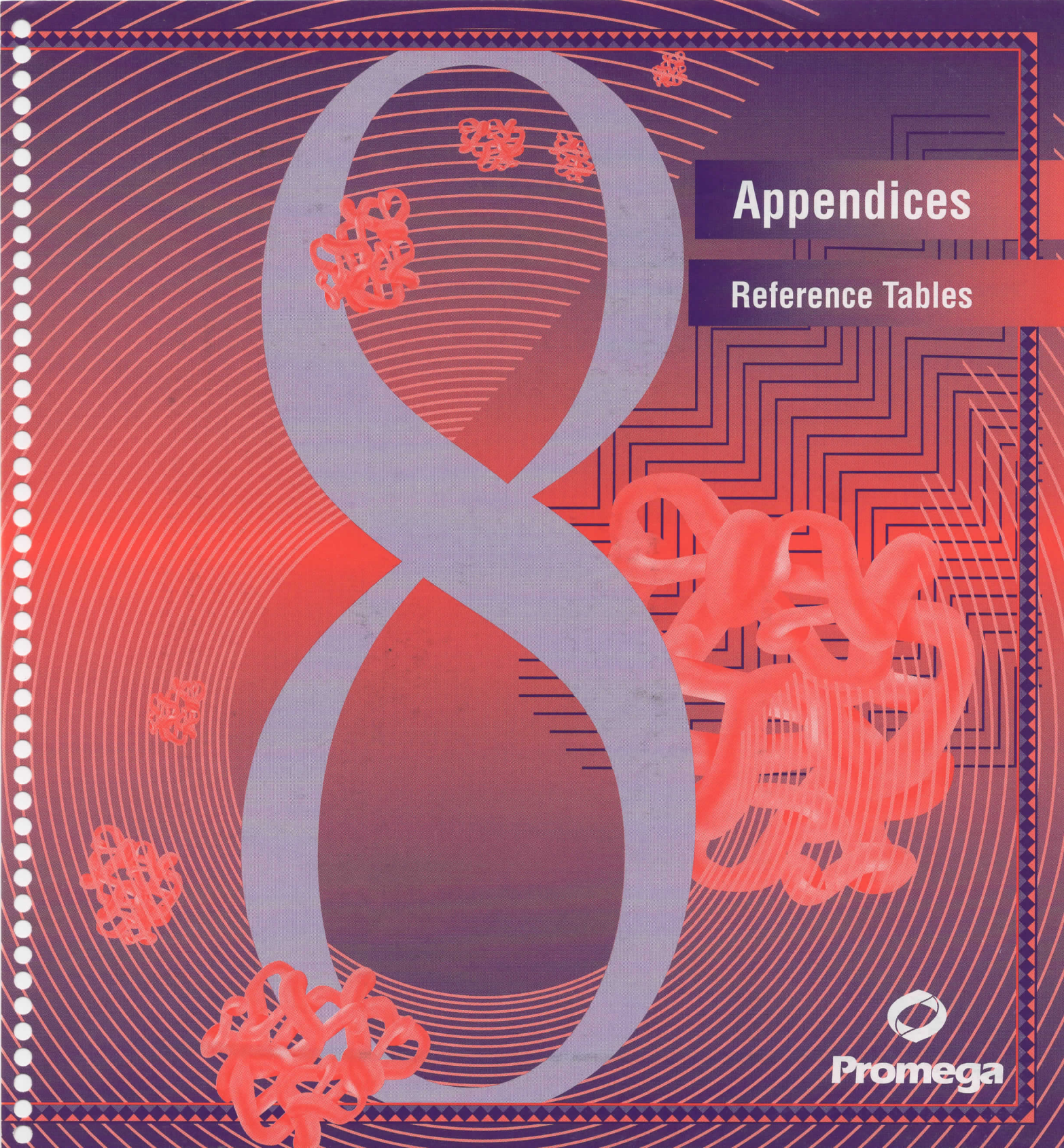

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Table 1. Common Conversions of Proteins.

Protein Molar Conversions

100pmols of 100,000 MW protein = 10µg
100pmols of 50,000 MW protein = 5µg
100pmols of 10,000 MW protein = 1µg

Protein/DNA Conversions

1kb of DNA = 333 amino acids of coding capacity = 3.7×10^4 MW
10,000 MW protein = 270bp DNA
30,000 MW protein = 810bp DNA
50,000 MW protein = 1.35kb DNA
100,000 MW protein = 2.7kb DNA

Table 2. Abbreviations and Molecular Weights for Amino Acids.

Amino Acid	Three-letter Abbreviation	One-letter Symbol	Molecular Weight
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine or aspartic acid	Asx	B	—
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic acid	Glu	E	147
Glutamine or glutamic acid	Glx	Z	—
Glycine	Gly	G	75
Histidine	His	H	155
Isoleucine	Ile	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

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Table 3. Codon Usage Table.

1st Position	2nd Position U	2nd Position C	2nd Position A	2nd Position G	3rd Position
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	**UAA** End	**UGA** End	A
	UUG Leu	UCG Ser	**UAG** End	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

The codons read in the 5′→ 3′ direction.
Termination codons are in bold.

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Appendix

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Table 4. A Brief Working Guide to Protease Inhibitors.

Name	Action	Stock Solution	Working Concentration	Comments
α_2-Macroglobulin (MW 725,000)	Almost all proteases; irreversible.	Aqueous. Stable at –20°C at pH 6.0-7.5.	Equimolar with protease.	Do not use in the presence of DTT. Sensitive to pH<4.
Antipain (MW 604.7)	Trypsin-like serine and some cysteine proteases; reversible.	10mM in aqueous solution; stable for 1 month at –20°C.	1-100µM; stable for several hours.	More specific than leupeptin for papain and trypsin.
Aprotinin (MW 6,500)	Serine proteases (not thrombin)	10mg/ml in H_2O; very stable at –20°C.	10µg/ml	Inactive at pH>12.8.
Bestatin (MW 308.4)	Aminopeptidases; reversible.	1mM in methanol; stable for 1 month at –20°C.	1-10µM; stable 1 day.	
Chymostatin (MW 604.7)	Chymotrypsin-like serine and most cysteine proteases; reversible.	10mM in DMSO; stable for months at –20°C.	10-100µM; stable for several hours.	
Cystatin (MW 12,000)	Cysteine proteases; reversible.	1mg/ml in 20mM Tris, pH 7.5. Stable to heat. Do not freeze.	100-500µg/ml	
E-64 (MW 357.4)	Cysteine proteases; irreversible.	1mM in H_2O; stable for months at -20°C.	1-10µM	
EDTA (MW 372.2)	Metallo- and metal-activated proteases; reversible.	0.5M in H_2O at pH 8.5; stable for months at 4°C.	1-10mM	Chelator. May interfere with other processes requiring metal ions.
Leupeptin (MW 426.6)	Trypsin-like serine and some cysteine proteases; reversible.	10mM in H_2O; stable 1 week at 4°C, 1 month at –20°C.	10-100µM	Good for Endoproteinase Lys-C, trypsin and papain.
Pepstatin A (MW 685.9)	Some aspartic proteases; reversible.	1mM in methanol or DMSO; stable for months at –20°C.	1µM	Binds weakly.

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Reference Tables

Table 4. (continued)

Name	Action	Stock Solution	Working Concentration	Comments
Phosphoramidon (MW 543.6)	Bacterial endopeptidases; reversible.	1mM in H_2O; stable for 1 month at –20°C.	1-10µM; stable for 1 day.	
PMSF (MW 174.2)	All serine proteases; irreversible.	200mM in ethanol; stable for months at 4°C.	0.1-1.0µM; Half life = 1 hr at room temperature.	Toxic. Prepare fresh.
TLCK (MW 332.5)	Trypsin-like serine proteases, irreversible.	10mM in methanol.	10-100µM; unstable above pH 6.0.	Prepare fresh.
TPCK (MW 351.5)	Chymotrypsin-like serine proteases; irreversible.	10mM in ethanol or methanol; stable several months at 4°C.	10-100µM; stable for several hours.	

Reference: Beynon, R.J. and Bond, J.S. (1989) In: *Proteolytic Enzymes: A Practical Approach*, IRL Press, Oxford.

Table 5. Temperature Dependence of pH for Commonly Used Buffers.

Buffer System	pK_a at 20°C	ΔpK_a/10°C
MES	6.15	-0.110
PIPES	6.80	-0.085
MOPS	7.20	-0.013
HEPES	7.55	-0.140
Tris	8.30	-0.310
Citric acid	3.10	
	4.75	
	6.40	
Phosphate	1.96	
	6.70	
	12.32	

Reference: Good, N.E. (1986) *Biochemistry* **5**, 467.

Table 6. Temperature Dependence of the pH of 50mM Tris-HCl Solutions.

4°C	25°C	37°C
8.1	7.5	7.2
8.2	7.6	7.3
8.3	7.7	7.4
8.4	7.8	7.5
8.5	7.9	7.6
8.6	8.0	7.7
8.7	8.1	7.8
8.8	8.2	7.9
8.9	8.3	8.0
9.0	8.4	8.1
9.1	8.5	8.2
9.2	8.6	8.3
9.3	8.7	8.3
9.4	8.8	8.5

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Table 7. Ammonium Sulfate Usage at 25°C.

Initial Percent Ammonium Sulfate	Final Percent Ammonium Sulfate Solution at 25°C																			
	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	111	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

This table provides the amount (in grams) of ammonium sulfate to add to a solution to adjust it to a new percentage. To determine the correct amount of ammonium sulfate to add, find the row for initial percentage of ammonium sulfate in the solution. Then move right along that row to the column containing the desired percentage. **Note:** A saturated ammonium sulfate solution is 4.1M (at 25°C) and requires 767g per liter. Ammonium sulfate solubility decreases by approximately 2.44g/°C.

Appendix

Reference Tables

Table 8. Ammonium Sulfate Usage at 0°C.

Initial Percent Ammonium Sulfate	Final Percent Ammonium Sulfate Solution at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	223	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20		27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25			27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30				28	57	86	117	148	181	214	249	285	323	362	402	445	488
35					28	57	87	118	151	184	218	254	291	329	369	410	453
40						29	58	89	120	153	187	222	258	296	335	376	418
45							29	59	90	123	156	190	226	263	302	342	383
50								30	60	92	125	159	194	230	268	308	348
55									30	61	93	127	161	197	235	273	313
60										31	62	95	129	164	201	239	279
65											31	63	97	132	168	205	244
70												32	65	99	134	171	209
75													32	66	101	137	174
80														33	67	103	139
85															34	68	105
90																34	70
95																	35

This table provides the amount (in grams) of ammonium sulfate to add to a solution to adjust it to a new percentage. To determine the correct amount of ammonium sulfate to add, find the row for initial percentage of ammonium sulfate in the solution. Then move right along that row to the column containing the desired percentage. **Note:** A saturated ammonium sulfate solution is 4.1M (at 25°C) and requires 767g per liter. Ammonium sulfate solubility decreases by approximately 2.44g/°C. (England, S. and Seifter, S. (1990) Meth. Enzymol. 182, 285.)

Technical Services:

800-356-9526

Promega

If you have technical questions regarding the content of this book, please contact our Technical Services Department at (800)356-9526.

Contributors

Gregory Beckler
Greg received a BA in Biology from SUNY-Plattsburgh, after which he studied at the W. Alton Jones Science Center. He then obtained a PhD from The Ohio State University, where he studied the molecular biology of methanogens and subsequently studied archebacteria prior to joining Promega. At Promega, Greg helped develop Flexi Lysate, the TnT Systems, the S30 Systems and the RiboMAX Large Scale RNA Production System.

Scott Breitlow
Scott received a BS from the University of Wisconsin-Madison. At Promega, he helped develop many of the protein stains available, such as Fast Coomassie and GhostBands Stains, and products for protein purification. Currently, Scott works as a Promega Field Application Specialist in the Houston area.

Martin Kline
Marty received a BS in Chemistry and Molecular Biology/Biochemistry from Purdue University. Marty previously worked at Sigma Chemical Company (St. Louis) in the Microbiology Department, where he developed large- and small-scale protein purifications and optimized fermentation yields. At Sigma, he also helped develop new clinical test kits for medical use. At Promega, Marty works on the purification of bacterial restriction enzymes with an emphasis on the scale up of these processes.

Gary Kobs
Gary received a BS in Bacteriology from the University of Wisconsin-Madison and worked for a number of years at the University studying RNA turnover. At Promega, Gary works in the Technical Services Department, where, in addition to answering customer questions, he has developed and presented technical workshops and training materials.

Gregg Larson
Gregg received a BS from the University of Wisconsin-Madison and worked at the University in protein chemistry research. At Promega, he has helped develop the proteases, clostripain and alkaline protease, the ChromaPhor Protein Recovery System, TMB Stabilized Substrate for HRP, the ProtoBlot II Systems and *Western Express* Protocol.

Scott Lesley
Scott received a BS in Microbiology from the University of Illinois and a PhD from the University of Wisconsin-Madison, where he studied prokaryotic transcription. Scott then worked on catalytic antibodies in the Chemistry Department at University of California-Berkeley. At Promega, Scott works on protein structure/function studies using *in vitro* translation and mutagenesis.

John Shultz
John received a PhD in Biochemistry from Purdue University. He subsequently did post-doctoral work at the NIH where he studied protein secretion mechanisms. He previously worked for Miles Laboratories studying protein expression and analysis. At Promega, John heads the Protein Biochemistry R&D Group and helped develop the ChromaPhor Stain, PepTag Non-Radioactive Protein Kinase Assays, Probe-Design System, Protein Fingerprinting System and sequencing grade proteases.

Carolyn Stock
Carolyn received a BA in Biology and a MS in Genetics from the University of Missouri-Columbia, where she studied filamentous phage. Prior to joining Promega, she worked at Agrigenetics Corporation developing gene transfer and expression systems for plants, and then at the University of Wisconsin-Madison as the developmental editor for the UWBC Biotechnical Resource Series. At Promega, Carolyn is a technical writer developing customer support literature.

David Thompson
Dave received a BS in Microbiology from the University of Wisconsin-LaCrosse. He previously worked at Agrigenetics Corporation in the DNA sequencing facility. At Promega, Dave has helped develop Sequencing Grade *Taq* DNA Polymerase, Altered Sites *in vitro* Mutagenesis System and the TnT Coupled Transcription/Translation Systems.

Doug White
Doug received a BA in Chemistry from the Illinois Institute of Technology and a MS in Biochemistry from the University of Wisconsin-Madison, studying protein kinases in *Paramecium*. At Promega, Doug has helped develop the PepTag Non-Radioactive Protein Kinase Assays, Protein Kinase C and other protein biochemistry products.

Promega Corporation

2800 Woods Hollow Road
Madison, WI 53711-5399 USA

Telephone	608-274-4330
Fax	608-273-6967
Telex	62057092
Australia	008 225 123
Austria	1 80105 324
Belgium (see Netherlands)	
Brazil	031 275 12 40
Canada	800 267 7424
Denmark	44 94 88 22
Finland	90 708 0900
France	05 48 79 99 or 1 45 33 67 17
Germany	06221 502122
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Switzerland	01 830 70 37
Taiwan	02 733 6877
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